GROWTH AND CELL PROLIFERATION KINETICS OF A MARINE PLASMACYTOMA IN RELATION TO ITS ENVIRONMENT

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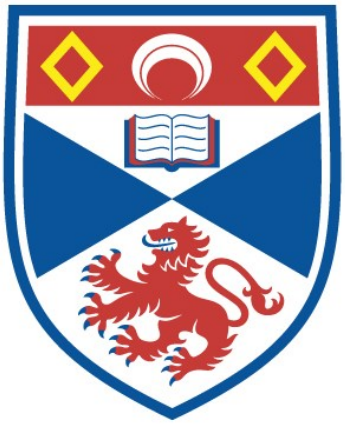

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GROWTH AND CELL PROLIFERATION KINETICS OF A MURINE PLASMACYTOMA IN RELATION TO ITS ENVIRONMENT.

A THESIS

Submitted to the University of St.Andrews for the degree of Doctor of Philosophy.

by

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October 1985 .

DECLARATION

I declare that this thesis is a record of my own work and that it has not been submitted for any other degree.

IHSAN K. CHALABI

St.Andrews,
October 1985 .

SUPERVISOR'S CERTIFICATE

I certify that Ihsan K. Chalabi, has spent 12 terms of research under my supervision, that he has fulfilled the conditions of Ordinance General No.12 and Resolution of the University Court 1967, No.1; and he is qualified to submit this thesis for the degree of Doctor of Philosophy.

Dr. A. C. Riches

St.Andrews,
October 1985 .

بسم الله الرحمن الرحيم

الحمد لله رب العالمين والصلاة والسلام
على سيدنا محمد وعلى آله وصحبه.
أهدي رسالة الدكتوراه هذه الى حضرة
سيدنا رسول الله صلى الله عليه وسلم،
ومن بعده الى والديّ والى اخي عرفانًا
لكل جوني وجهلني منهم على مدى سنيّ
دراستي هذه .. ولولاهم ماكان لهذا البحث
ان يُنجز .

In the Name of God the Most
Merciful the Most Compassionate.

Thanks be to God Almighty for his grace and help in the accomplishment of this dissertation.
I dedicate my work to my master the prophet Muhammad (peace be upon him), to my parents and to my brother in gratitude to the invaluable help they extended to me, without which, this research would not have been accomplished.

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ABBREVIATIONS

B cell = bone marrow derived lymphocyte

Ca^{2+} = calcium ion

°C = degrees centigrade, (celsius)

CFU-s = colony forming unit-spleen

ci = curie

CO_2 = carbon dioxide

cpm = counts per minute

DNA = deoxyribonucleic acid

EDTA = ethylenediamine tetraacetic acid

FCS = foetal calf serum

Gy = grey

Hepes = N-2-Hydroxyethyl-piperzine N'-2-ethanol sulfonic acid

3H-Tdr = (methyl-^{3}H)Thymidine

3H = ^{3}H

H3 = ^{3}H

ID50 = dose of polyamine per culture that produces a 50% inhibition of isotope uptake by cell cultures.

Im = mitotic index

Imet = metaphase index

in^2 = square inch

Ip = growth fraction

Is = labelling index = LI

IUDR = iodo-deoxyuridine

125IUDR = 5-(^{125}I)iodo-2'-deoxyuridine

KB = birth rate

KG = growth rate

KL = cell loss rate

Kv = kilovolt

LDL-In = low density lipoprotein inhibitor

ln = natural logarithm = $\log_e$

$\log_{10}$ = common logarithm

ma = milliampere

mci = millicurie

mg = milligram

Mg^{2+} = magnesium ion

MHC = major histocompatibility complex

ml = milliliter

mm^3 = cubic millimeter

m mole = millimole

MPC = murine plasmacytoma

MTC = mouse thymocytes

m .wt. = molecular weight

NaCl = sodium chloride

NaOH = sodium hydroxide

NH_4Cl = ammonium chloride

NK = natural killer cell

PBS = phosphate buffered saline

PC = plasmacytoma

PFC = plaque forming cell

pH = negative logarithm of the hydrogen ion concentration

RNA = ribonucleic acid

RPM = revolutions per minute

RTC = rat thymocytes

sp. = specific
sp. act. = specific activity

SRBC = sheep red blood cells

S.E. = standard error

T cell (lymphocyte) = thymus derived lymphocyte

Tc (lymphocyte) = cytotoxic lymphocyte
TcL(lymphocyte) = cytotoxic lymphocyte

Tc = median inter mitotic (cell cycle) time

Tca = apparent cell cycle time

tm = duration of mitosis

ts = duration of S phase

um = micron

uci = microcurie

UIF = uptake inhibitory factor

uM = micromole

$>$ = greater than

$\geq$ = greater than, or equal to

$<$ = less than

$\simeq$ = approximately

$\sum$ = sum of

CONTENTS

CHAPTER ONE

INTRODUCTION

Before describing the experimental work in this study, an introduction to the kinetic terms and concepts and the classification of cell populations is appropriate.

1.1.1 The cell cycle:

The cell cycle is usually considered to begin with the completion of one cell division and to end with the completion of the next division. An important marker of kinetic events and cellular proliferation before the studies of Howard and Pelc (1953) was mitosis. Progress through the cell cycle may be assessed by observing two stages, that of mitosis, and of DNA synthesis as shown by Howard and Pelc (1953). Howard and Pelc (1953) suggested that the cell cycle could be represented in terms of progression around a clock face, divided into four successive intervals, Gap 1 (G_1), S (DNA synthesis), Gap 2 (G_2) and mitosis (M). G_1 and G_2 are gaps during which no DNA synthesis occurred.

The G_1 phase:

The G_1 phase of the cell cycle is important with regard to the control of cellular proliferation. This is because the cell upon

reaching G_1 has the option to continue in the proliferative cycle, or leave the proliferative cycle (decycle). A probable control point in this phase is the initiation of DNA synthesis. RNA and protein synthesis are required in G_1 for the initiation of DNA synthesis, (Baserga 1976, Prescott 1976). The progress of cells in G_1 is divided into 2 distinct compartments G_1A and G_1B, cells in G_1A can not progress directly to S phase before passing into G_1B. The transition into G_1 B is accompanied by an increase in RNA above a certain level, and above this level of required RNA content the cells move to S phase, (Darzynkiewicz et al., 1980). Liskay et al., (1980) have used the V 79-8 chinese hamster cells which lacked a G_1 phase under normal conditions. When the amount of protein synthesis was decreased with cycloheximide, the flow of cells into S phase was delayed. The generation time increased by 5 hours, S, G_2 and M phases remained relatively stable, and G_1 phase was acquired. The amount of DNA in G_1 cells is half that of cells in S phase, where the DNA is duplicated. The cytoplasm is important in the initiation of DNA synthesis, in particular the ratio of cytoplasm to nucleus, a higher cytoplasm to nucleus ratio, initiates G_1 cells for DNA synthesis, (Prescott 1976). Specific molecules are also associated with growth and initiation of DNA synthesis, acting as positive or negative control mechanisms for entry into S phase

Yanishevsky and Stein (1981) have shown that fusion of Hela cell nuclei in certain binucleate combinations from early, middle and late G_1 cells; resulted in acceleration towards S phase; the highest contribution in acceleration was by late G_1 nuclei and the smallest was by early G_1 nuclei.

Baserga (1978) used temperature sensitive mutants to assess the potential of G_1 blocked cells in initiating DNA synthesis. Temperature sensitive mutant cells (ts) arrested in G_1 at the non-permissive temperature were fused to chicken erythrocytes; the (ts) mutants could not initiate DNA synthesis in the erythrocytes, while (ts) mutants used at the permissive temperature did initiate DNA synthesis in the erythrocytes. Similarly, Baserga (1978) has used cytoplasmic extracts from cells blocked in G_1 at the non-permissive temperature and cycling cells. Extracts from G_1 blocked cells could not initiate DNA synthesis in frog nuclei, while extracts from cycling cells initiated DNA synthesis.

Yanishevsky and Stein (1981), have demonstrated in three cycling cell types, one normal and two transformed, which could not induce DNA synthesis in senescent HDC cells, when fused to them; rather, the senescent HDC cells impaired the ability of the cycling cells to enter S phase. The fusion experiments may show that the entry into S phase in cycling cells is positively controlled together with a negative control mechanism available in resting cells.

The S phase:

At the end of G_1 phase, enzymes associated with DNA synthesis start to rise. At the G_1-S boundary, the DNA polymerases necessary for DNA synthesis are active, and in S phase DNA and chromsomal proteins are duplicated. Proteins and RNA syntheses are required for DNA synthesis, (Baserga 1976, Prescott 1976). In vitro, S phase serves as a marker for cells using radiolabelled nucleotides, in autoradiography and other techniques that make use of

radioactive substances. Proliferating cells are sensitive to various inhibitors which can affect DNA synthesis in in vitro cultures and this will be discussed later.

The G_2 phase:

The G_2 phase is of short duration and is a period for preparation to mitosis. Cells may arrest at G_2phase as shown by Gelfant (1977, 1981). Protein synthesis in G_2 is required for mitosis, (Sisken and Iwasaki 1969). RNA is also required to complete $G_2$and enter mitosis, (Kishimoto and Liebermann 1964).

Mitosis:

In this phase, the segregation of chromosomes occurs, after which, the cell divides into two new individual cells. The process of mitosis is divided into four phases: prophase, metaphase, anaphase and telophase.

In mitosis most of RNA synthesis ceases before metaphase is reached, and the rate of protein synthesis also drops with exception of chromosomal proteins (Baserga 1976, Prescott 1976).

Regulation of cell production is attained by causing the cells to arrest in G_1 and G_2,(Epifanova and Treskikh 1969) and in S phase (Darzynkiewicz et al., 1980). The G_1 phase in a homogeneous population is more variable than S $G_2$and M phases, (Sisken and Kinosita 1961). The variableness in the length of the G_1period in cells gives rise to most of the variability in generation times

within a cell population. It may be that the variability represents a variable rate with which the cells may move along the whole or part of the G_1 phase; or the cells may be transiently arrested for various time periods. The arrest in the G_1 and transition to G_0 and its reversibility may reflect a mechanism by which cell reproduction is regulated (Prescott 1976).

Environmental changes that may account for different generation times in CHO cells grown in various sera, gave different generation times for each type of sera. The difference in generation times was accounted for by the changes in the average duration of G_1, with small changes in S, G_2 and M phases, (Tobey et al., 1967). The frequent trend among normal or tumour cells is a variable G_1 period (Prescott 1967), although the example of Lala and Patt (1966) showed a constant G_1 in the ascites tumour cells. The variability in G_1 may also relate to cell size, (Killander and Zetterberg 1965), (Yen et al., 1975), (Precott 1976), (Yanishevsky and Stein 1981). The variability in the length of G_1 may be due to the variability in the cell's mass at the start of G_1 phase. Daughter cells produced at mitosis may not be equal in size, small sized daughter cells take a longer time to reach a critical division size than larger cells. i.e. sizer model effect, (Fantes and Nurse 1981). Fournier and Pardee (1975) have shown that cell size and mass did not influence the passage of cells in G_1, although Yen and Pardee (1979), have shown that cells with small nuclei after mitosis take longer to reach S-phase than cells with larger nuclei.

In vitro the cell is responsive to changes in the extracellular environment such as nutrients, serum, pH and cell density, and the cell may arrest in G_1 if the associated changes are

unfavourable to its proliferation, (Pardee 1974), (Pardee and Dubrow 1977), (Pardee et al., 1978). Pardee (1974) has introduced the term restriction point to describe the reversible arrest of cell proliferation at the same point in G_1 due to suboptimal conditions of nutrients. Thus the cells due to unfavourable environmental conditions may decycle at this point. Non specific regulation influencing the cell cycle may be due to the unfavourable conditions like deprivation of nutrients required by the cells. Non specific regulations can be seen in unicellular organisms or in vitro cultures; but in multicellular organisms where environmental and nutrient conditions are usually favourable, the regulation of cell production may be acieved by humoral mediators, such as hormones or chalones. Thus nutrient restriction and specific regulations of cell production may influence proliferative behaviour of cells.

1.1.2 The phase of proliferative rest. Cycling and noncycling cells.:

In an ideal cell population where all the cells in a population are continually cycling, with no cell loss imposed upon them; the cells will double their number after completing their transit through all the phases of the cell cycle in one cell cycle time (Tc). This is an unusual situation in vivo where some cells are not engaged in the cell cycle; the cells may be in a state of proliferative rest or are dying. Some resting cells are able to enter the cell cycle when responding to environmental stimuli. The state of no cycle or dormancy (D-state) was described by Lajtha et al., (1962), and there after this no cycle state has been referred to as a G_0 state by Lajtha (1963), Gilbert and Lajtha (1965), Lajtha

and Gilbert (1967) and Lajtha (1983). The putative G_0 state is an out of cycle resting state under normal physiological conditions, and acts as a reservoir to keep the tissue in its optimal size, when the tissue is subject to cell depletion or injury. A G_0 hepatocyte has a DNA content similar to a G_1 cell, and in partial hepatectomy the G_0 are triggered back into cycle with a delay of around 15 hours to reach S phase in a wave like pattern, followed by a wave of mitosis. If these triggered hepatocytes were in a lengthy G_1, then the expected shortening of time (to 15 hours) would reflect an immediate increase in DNA synthesis and mitosis, (Lajtha 1963). Following Osgood's (1957) description that a cell upon division yields an immature cell and another cell for the differentiation pathway, Cairnie et al., (1965) used the terms P and Q to describe proliferating cells and non proliferating quiescent cells. The populations of cells suggested by Lajtha et al., (1962) and as reviewed by Epifanova and Terskikh (1969) would be composed of a resting population and a proliferating-differentiating population which is supplied from the self maintaining resting population, which will be triggered into cycle to supply them. Apart from proliferative rest at G_0, Epifanova and Terskikh (1969) have shown that cells may arrest also at G_1 and G_2. Quiescent cells may be a distinct out of cycle state (Lajtha 1963), (Baserga 1976-1978), (Pardee 1974), (Pardee et al., 1978), or quiescent cells may be in a very extended G_1 (Prescott 1976), and no requirement for G_0 compartment was described by Shields and Smith (1977).

Burns and Tannock (1970) proposed that the cell cycle has 2 phases, G_0 phase, and the C phase which consists of part of G_1, S, and G_2 and mitosis. The cells leave G_0 to the C phase with a constant probability per unit time. Smith and Martin (1973-1974)

proposed a similar description to that of Burns and Tannock (1970), the cell cycle is divided into a probabilistic A-state and a deterministic B phase; the B-phase consists of part of G_1, S, $G_2$and mitosis. That is after mitosis all cells go into the A-state for any length of time, after which they progress towards B phase.

The transition probability (P) is fixed, although it may change as the environmental conditions change, and it may vary according to cell type. The transition probability model is compatible with the view that quiescent cells are cycling slowly, because it suggests that quiescent cells are in fact cycling G_1 cells with a low transition probability. The transition probability may explain that the variability in generation times is due to the probabilistic transition from the A state to the B phase, (i.e. the cells are being held in a state of no progress towards mitosis), being part of the cell cycle in G_1phase; the cell will remain in the A-state for any period of time, but will leave the A state exponentially with respect to the time they have spent in the A-state.

A further addition to the transition probability model was described by Brooks (1981), (see Figure A.) to take into account some of the experimental variables which are connected with the cell's lag duration when stimulated to proliferate and the commitment event into DNA synthesis; and also to take into account the similarity of time durations of the lag period and the minimum cycle time (T_c) of B phase.

The model also shows that the differences in cell cycle times between sister cells can be described in terms of an exponential as the Q ---> L part of the sequence occurs in the mother cell and thus is common to the two sister cells, to account for the identical

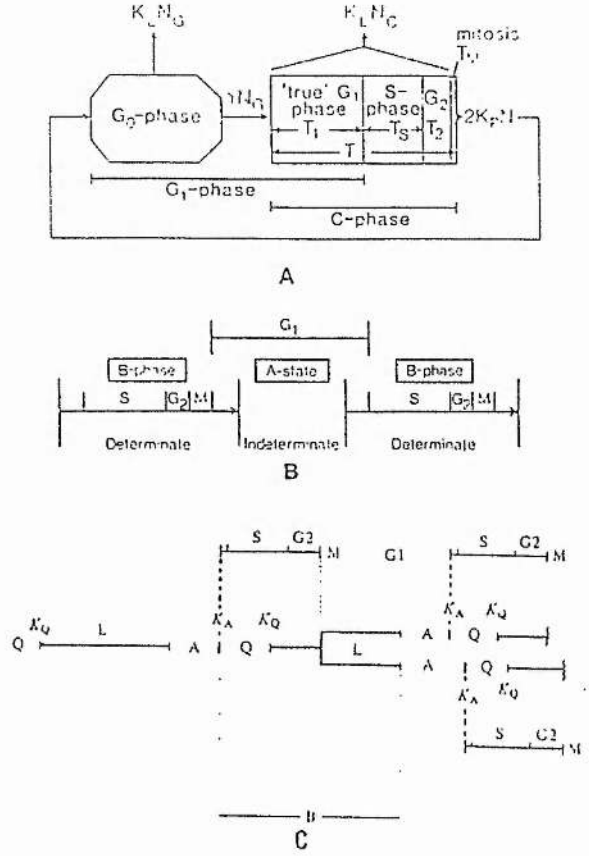


Fig. A: Alternative models of the cell cycle; (A) Burns & Tannock (1970); (B) Smith & Martin (1973); and (C) the modification introduced by Brooks (1981).

B-phase in sister cells. The cell cycle therefore, according to Brooks (1981) contains two random transitions and that the quiescent cells are located not in the A state but in another indeterminate state called Q. In order to pass from Q to A state, cells must complete a process L which occupies most of the lag period. The cells in progress towards division leave the Q-state with a random transition and start the L-process, then after completing the L-process the cells go into A-state. Cells in the A-state, will leave this state with a certain transition probability to DNA synthesis passing through the Q-state and L-process.

Small quiescent cells take a longer time to reach S phase than large cells. It is possible that both deterministic and probabilistic models account for cell cycle controls (Nurse 1980). If in a cell the accumulation of subunits and assembly of structure required for mitosis is cell size dependent (deterministic size related mechanism), smaller cells require more time than larger cells. Upon completion of the structure, the iniation of mitosis involves an interaction between a certain cellular component and the structure, and this may be a random process, forming the probabilistic element in control. If the formation of materials required for the structure is slow, the assembly of the structure will be the rate limiting factor in the cycle, division control is consistent with size control. i.e. deterministic control of division. If the formation of substances and their assembly is rapid, then the event limiting the rate of cell cycle progress is consistent with transition probability model; (Nurse 1980, Fantes and Nurse 1981). Darzynkiewicz et al., (1980) have shown that the rate of cell exit across two subcompartments in G_1(G_1A-->G_1B) correlates with their RNA content, thus Darzynkiewicz et al.,

(1980) have concluded that the relationship between RNA and the position of cells in the cycle may show that the indeterminate A-state is not the sole factor responsible for large intercellular variations in generation times.

The view that cycling cells and resting cells may be different in character was shown by Sander and Pardee (1972) on nutrient deprived cells in cultures. The time required by the isoleucine arrested cells to reach S phase after adding isoleucine was longer than the time required by cells reaching S phase directly from mitosis. i.e. G_1 is longer in resting cells that were stimulated into cycle, than normal cycling cells.

Further evidence for the G_0 state of rest was suggested by the work of Augenlicht and Baserga (1974). They showed that when WI-38 cells were cultured in vitro, up to the stage of confluence, the cells will arrest in G_1; thereafter the cells will shift into a different state of arrest as the period of rest increases after reaching confluence. This was proved by stimulating resting cells with serum at different periods of time after reaching quiescence; the longer the cells remain quiescent, the fewer cells and a longer pre-replicative period before reaching S phase after restimulation with serum; i.e. a deeper G_0 state is developing in the quiescent cells, (Baserga 1976-1978). Potter (1978) has extended the G_0 idea of Lajtha (1963) by stating that cells after mitosis can shift to G_0 and can exist in a variety of G_0 states with a terminal state G_T of differentiation, followed by death. The different stages of the G_0 phase are associated with intermediate or new stages of differentiation, a G_0 cell at any point before reaching the G_T state, can recycle again into G_1; whereas upon reaching G_T the cell can not recycle. Apart from arrest and cell cycle regulation in G_1, cells

may arrest in S phase (Martin and Oppenheim 1977), (Darzynkiewicz et al., 1980), and G_2 phase (Gelfant 1977, 1981).

Different states of rest were shown by Radley et al., (1976) who have demonstrated in the acinar cells of that rat salivary gland which were stimulated by isoproterenol twice and by a third stimulation after 28, 52, 76 hours, reflected a correlating increase in the time period to reach DNA synthesis of 16.2, 20.9 and 25.6 hours respectively. Another mechanism linking the state of rest and proliferation was demonstrated by Alison and Wright (1979a) who have assessed the labelling and mitotic indices in the seminal vesicle of castrated mice at 3, 9, and 14 days after castration. The mice received testosterone injections at these specified time periods. The prereplicative period between quiescence and DNA synthesis gets progressively shorter, as the interval between castration and testosterone adminstration gets longer (Alison and Wright 1981).

Alison and Wright (1979a, 1981) have proposed that there was a shallower level within G_0 as the period of physiological quiescence gets longer with respect to androgen deprivation. Terskikh et al. (1981) have described their in vitro results as similar to those of Augenlicht and Baserga (1974). Wallen et al., (198a,b) in their studies on Q--> p recruitment, have shown that when mammary tumour cell lines were cultured to a plateau level, and when the cells were recultured again at various intervals after reaching the plateau level, the time to reach DNA synthesis increased as the period between the plateau level and the reculturing increased. This was also reflected in an exponential decline in the colony forming efficiency as a function of time spent in culture. Wallen et al., (1984b) remarked that these results may represent a

situation similar to that of Augenlicht and Baserga (1974), or may have a more probable explanation, that these cells were not progressing into a deeper G_0 state but that the cells in the Q state observed in these tumour cell lines were progressing towards death. As the cells move away from the P state fewer cells can make the Q-->P transition; although not all the cells that make the transition are clonogenic. The clonogenicity of all the cell lines tested decreased, but at those time periods when the cells were subcultured for colony efficiency analysis, they were able to proliferate when subcultured for proliferation assays.

Epifanova (1977) has defined a resting cell as a cell withdrawn from the mitotic cycle for an indefinite time interval surviving in this state and retaining the potential for proliferation. This implies that in the resting state a cell loses some of its characteristics of a proliferating cell, being in a different physiological state with special metabolic patterns. Epifanova et al., (1980) have stated that there were processes regulating the transition of cells from proliferation to rest and vice versa, that cell proliferation needed some metabolic processes to be suppressed and some processes to maintain the viability of resting cells. Polunovsky et al., (1984) have shown in their experiments that the fusion of resting cells (serum deprived NIH 3T3), with proliferating (serum stimulated) cells, inhibited the onset of DNA synthesis in the proliferating cell's nucleus. This indicated a negative control on cell proliferation via an endogenous inhibitor that prevented the onset of DNA synthesis. Resting cells produced an endogenous inhibitor of cell proliferation whose formation depended upon synthesis of protein. Upon stimulating resting cells, proliferation can start only after decreasing the level of the inhibitor. Cells grown to high cell

density stationary phase contain an inhibitor of protein synthesis, that was not detected in proliferating low density cell cultures, (Englehardt 1971).

A variety of differences between resting and growing cells have been reported. Evans et al., (1974) have shown that there was more microvilli on the surface of actively growing 3T3 fibroblasts, compared to a smooth surface with fewer microvilli in resting cells. There is also a decreased permeability to low molecular weight substances in resting cells such as amino acids, sugars and phosphates, (Pardee et al., 1978). Putrescine transport is also reduced in resting human fibroblasts as compared to growing fibroblasts, (Pohjanpelto 1976). The concentration of adenine nucleotides in stationary phase cultures of the WRL-10A cell line was about half the amount of that available in actively growing cultures; (Glinos and Warren 1980). Wallen et al., (1984a) have shown that the quiescent cells arrested in G_1 were 50% smaller in size than proliferating cells and the RNA content decreased by more than half the amount to that available in proliferating cells. Sander and Pardee (1972) have shown that differences existed between resting cells in G_0 and cycling cells. In nutrient deprived arrested cells, the uptake of 2-amino-isobutyric acid did not increase for a 3 hour period after stimulating the arrested cells with isoleucine; while in cells cycling normally from mitosis to S phase the uptake of 2-amino-butyric acid doubled within half an hour after entering G_1 from M phase. The time required to reach S phase was longer in stimulated cells than in cells cycling normally from mitosis to S phase; i.e., G_0 are different to early G_1 cells as shown by the longer pre-replicative period and time for uptake of 2-amino-butyric acid.

Farber and Baserga (1969) have shown that after stimulating the salivary glands and kidney cells with appropriate stimuli, and hepatocytes after partial hepatectomy, the stimulated cells that were triggered to flow to DNA synthesis were not killed by hydroxyurea, although hydroxyurea was cytotoxic to normal cycling cells of the jejunum and tongue epithelium. A small fraction of the isoproterenol stimulated cells were necrotic because of the action of hydroxyurea. Ara/c and nitrogen mustard were also tested and were effective in killing cells that were in normal cycle, and not the isoproternol stimulated cells.

The same differential cytoxicity between stimulated resting cells of the seminal vesicle and cycling cells of the crypts in mice in terms of the effects of Ara/c and hydroxyurea were described by Alison and Wright (1979b, 1981).

Cress and Gerner (1980) and Magun and Gerner (1981) have found that the induction of ornithine decarboxylase (a rate limiting enzyme in polyamine biosynthesis) in dividing cells may be under different regulations as compared to quiescent cells. Hydroxyurea inhibited the induction of ODC in dividing cells, but did not inhibit the induction of ODC in quiescent cells stimulated to proliferate. Darzynkiewicz et al., (1979) have found differences between quiescent and stimulated lymphocytes, the chromatin in quiescent cells was more sensitive to acid denaturation than the DNA in cycling interphase lymphocytes. The former has a higher red shift in acridine orange-DNA complex, while in the latter there was a corresponding green shift. Darzynkiewicz et al., (1981) reported some differences between quiescent lymphocytes (G_0) and PHA stimulated lymphocytes in response to uptake of rhodamine 123. Nonstimulated (G_0) lymphocytes show low and uniform value of

rhodamine 123 flourescence, while in the PHA stimulated lymphocytes the uptake of rhodamine increases several folds. Riddle and Pardee (1980) have demonstrated that there was an enhanced actin synthesis in cells stimulated into cycle by the addition of serum, relative to the actin synthesis in normally cycling cells, while some three other proteins were higher in cycling cells than in quiescent cells. Higher actin synthesis may represent a process that these cells may perform before entering G_1.

The criteria for the various resting and stimulated cell populations has been discussed by Gelfant,(1977). Resting cells are present in tumours and they are held back from proliferation by lack of essential nutrients and some homeostatic mechanisms, but they are able to recycle when conditions are favourable (Steel 1977). Kallman et al., (1980) have described the proliferative classes of cells in a tumour: proliferating (P) cells in active cycle and have clonogenic potential; P cells can produce 2 P cells, one P and one Q cell or 2 Q cells. There is also a p-->Q transition without division. The Q cells are composed of Q undifferentiated and noncycling, and a Q maturing differentiating cell. The Q cells are available because of environmental factors restricting their proliferation and may recycle, or can feed the Q compartment. In the Q compartment, some non-cycling cells in well differented tumours may be withheld from proliferation because they have not escaped completely from the homoestatic mechanisms regulating the tissue of origin.

The Q cells can recycle and have been shown to produce colonies like P cells by Barendsen et al., (1973), Hermens and Barendsen (1978), Kallman (1980) and Wallen et al., (1984). The Q cells can also repopulate a tumour after cytotoxic treatment as

shown by Stephen and Steel,(1980). The transitions between the cycling and noncycling states in tumours and normal tissues can be viewed as a proliferative ecosystem which confers adaptive survival to the tissue and which allows for a variety of tissue proliferative responses,(Gelfant 1981).

1.1.3 The growth fraction:

Mendelsohn,(1960,1962) observed that in tumours there was a higher frequency of thymidine labelling among mitotic figures than among cells in the tumour; and suggested that tumours may be heterogeneous in terms of proliferation due to a mixture of cycling and non-cycling cells, (Mendelsohn 1963, 1965). Mendelsohn developed a relation for cycling and non-proliferating cells and termed it the growth fraction (G.F or Ip)

$$GF = \frac{\text{labelled cells/100 (labelled + unlabelled) cells}}{\text{labelled mitosis/100 (labelled + unlabelled) mitosis}}$$

$$Ip = Nc/N$$

Ip is the proliferative index (Growth fraction), Nc is the number of cycling cells, and N is the total number of cells in the population, (Aherne et al., 1977). Therefore assuming a cell population that has no cell loss and has some cells which were not proliferating; the time taken to double the size of the population will be longer than the median intermitotic time of the fraction of the proliferating cells in the population. This extended period of time to double the population size is the potential population doubling time (Tpd); (Steel and Bensted 1965), (Steel 1968, 1977); and is also called the apparent cell cycle time (Tca); (Wright 1975), (Aherne et al., 1977).

The cell production rate is determined by the growth fraction and the cell cycle time of the proliferating cells. When a growth fraction is present, it indicates that some cells are decycling and some cells are entering the cell cycle. Bresciani,(1968) proposed a relation (the distribution ratio) between cycling and decycling cells. The distribution ratio = $\overleftarrow{n}/\overrightarrow{n}$, that is $\overleftarrow{n}$ is the proportion of cells in cycle or recycling cells and $\overrightarrow{n}$ is the proportion of cells decycling to the quiescent non-proliferating compartment. The ratio between the number of cycling and decycling is associated with Ip: when $\overleftarrow{n}/\overrightarrow{n} > 1$, there is expansion in growth.

The d-ratio of Bresciani (1968), was developed further by Appleton et al., (1973) into the decycling probability, described by its effect on the growth fraction, and as a computer simulated model for G_0 hormone stimulated cells that have various probabilities of decycling.

1.1.4 The classification of cell populations:

In cell population studies it is necessary to classify the population in terms of morphology, anatomical site and proliferative ability. For example, in the crypt of Lieberkuhn of the small bowel mucosa, mitotic activity is available in the lower parts of the crypt. The lower part of the crypt is the proliferative zone or compartment which supplies cells to the villus which is the functional compartment. In the bone marrow spatial compartments are not as well defined as in the small bowel mucosa, the bone marrow cells are defined in terms of their morphology, as they progress through their differentiation pathway from stem cells to mature cells.

Leblond et al., (1959) have proposed a classification of mouse and rat tissues, based on the extent to which they take up 3H-thymidine into their DNA. The percentage of labelled nuclei was estimated in the tissues of adult mice sacrificed 8 hours after 3H-thymidine injections (cell formation test), and rats given 3H-thymidine when 3 days old, and sacrificed 6 months later (cell retention test). The results led to a classification of the cell populations into 3 groups:

Group 1: stable cell populations, which showed no cell formation in the adult animal, but in young rats the label was taken up when cells were proliferating, and was retained thereafter in the cells that remained in the population. This group includes smooth and voluntary muscles and neurones.

Group 2: growing or expanding cell populations, cells were capable of both cell proliferation and cell retention of the label in adult life. This group included liver parenchymal cells, adrenal cortical and medullary cells.

Group 3: renewing cell populations, cells showed many labelled nuclei in the cell formation test, but none in the cell retention test, the cell population was in a steady state, proliferating and losing cells continuously. The tissues in this group included the epidermis, bone marrow and small bowel mucosa.

Gilbert and Lajtha, (1965) proposed to characterise cell populations in terms of the relative importance of 3 parameters: the rates of cell input, output and cell division or production; and accordingly classified cell populations in terms of compartments that considered the above mentioned parameters.

Aherne et al., (1977) have described the classification of cell populations, and their compartmental system as follows:

1. Closed static compartment: in which there was no renewal of cells and a negligible loss, e.g. the neurone population of the central nervous system.

2. Decaying compartment: there was no renewal of cells, and output exceeded input, e.g: adult ovary.

3. Stem cell compartment: proliferating self renewing with output but no input, and supplies cells to other compartments, e.g.: basal layer of the epidermis.

4. Simple transit compartment: a functional compartment in which cells cease to proliferate. This compartment receives cells from the stem cell compartment, and outputs the cells eventually to their death pathway; e.g. cells in the upper portion of the small bowel crypts.

5.Dividing transit compartment: such cells are found in the bone marrow and the proliferative portion of the small bowel crypt. There is input from the stem cell compartment, which has an output to feed the maturation comparment.

6. Closed dividing compartment: there is no output or input of cells. There will be an increase in size in this compartment, as cells which proliferate are retained. Examples for this group are the liver cells regenerating after partial removal, proliferating tissue cultures and tumours in which its assumed that they have no cell loss .

In tumours and growing normal tissues, cell production exceeds cell loss, and are therefore expanding populations. In the adult, renewing cell populations maintain a constant size; i.e.: cell production equals cell loss, thus the tissues are in a steady state.

1.1.5 Age distributions of cell populations:

Most cell populations in vivo are not synchronised, but have cells that are distributed in terms of cell age and phase. The age structure or distribution of cell ages, is necessary in calculating the growth of a population; it is a relationship between the number of cells in a phase of the cell cycle and the duration of that phase. There is an expanding distribution (exponential) and a steady state distribution, (Lala 1971), (BrynmorThomas 1974), (Steel 1977), (Aherne et al., 1977). When all the cells in a population are expanding in multiples of their numbers and their cell cycle time (Tc), with no cell loss, it is expected to conform to theoretical exponential growth. In real situations there is exponential growth, but coupled with cell loss. In the steady state, which has a constant population size with time, there is a rectangular distribution, where cell production is balanced by cell loss, and the number of cells in each phase is proportional to the time spent in that phase.

Therefore in steady state the mitotic index or the proportion of cells in mitosis = Im = tm/Tc

The labelling index or the proportion of cell in DNA synthesis = LI or Is = ts/Tc

The mitotic rate = Im/tm = kp cell production rate or KB (birth rate) (cell loss assumed to be insignificant at mitosis)

In terms of cell cycle time, Im/tm = 1/Tc = KB

and in terms of the labelling index,

KB = LI/ts

In the expanding exponential populations the cells are not distributed in proportion to their phase durations. The proportion of young cycling cells (a) produced, are highest at t = o, after mitosis, and their number falls gradually as cell age (t) approaches the next mitosis. Thus the rate of entry into S phase is greater than into mitosis.

The mitotic index, therefore, when all cells are in cycle:

Im = In2 tm/Tc (Smith and Dendy 1962), (Cleaver 1967), (Lala 1971), (Aherne et al., 1977)

and

LI or Is = [exp(ts ln2/Tc)-1][exp t2 ln2/Tc]

In a uniform population of cells, producing 2 proliferating cells per division (a=2), a growth fraction of unity, and no cell loss; the population will double its number per each cell cycle time Tc

Nt = No exp ln2.t/Tc (Nt = the number of cells after an elapsed time and, No = initial number of cells before growth).

If some proportion of the daughter cells do not recycle ($\overleftarrow{n}$) into the proliferative (P-compartment) but decycle ($\overrightarrow{n}$) into the quiescent [Q-compartment], the proportion of cycling cells (a) lies between 1 and 2. The time to double in size will be longer, the growth fraction being less than one.

GF or Ip = a-1 (Lala 1971), (Steel 1977)

The cell population with a growth fraction and no cell loss, in which each cell entering mitosis, 'a' cells are born and 'a-1' cells are added to the population, two types of new cells are obtained.

No(a-1) new proliferating cells (cycling)

No(2-a) new non-proliferating cells (decycling)

and the

GF = New proliferating cells/all new cells.

The age distribution will be influenced by the value of 'a' when the growth fraction is less than unity. If loss were random with respect to cell age or proliferative status, the age distribution will not be affected. If cell loss were selective, with respect to cycle phase or compartment, for example, at

mitosis, "a" will decrease. The age distribution will be influenced by a small "a" and high rate of cell loss, to the extent that an exponential distribution is better described as a rectangular distribution.

1.1.6 Cell birth and loss rates:

The average birth rate is controlled jointly by the average cell cycle time and the growth fraction, thus:

1.

In steady state, KB = Ip/Tc = rm (rm = rate of entry of cells into mitosis) and,

2.

In exponentially growing populations:

$$KB = \ln (1+Ip)/Tc = rm$$

and assuming no cell loss, the growth rate of the population (KG) will reflect the birth rate KB. If the growth rate value was less than the expected birth rate, then the difference between the two rates is a measure of the rate of cell loss (KL)

$$KL = KB-KG$$

The growth rate in solid tumours can be measured in terms of tumour weight or volume. Assuming exponential growth at early tumour age, a tangent can be drawn to the growth curve, the growth rate (KG) can be found at any point from the slope of the tangent. Because with increasing tumour age, associated with extensive cell loss and low growth fraction, the distribution is unlikely to be exponential.

For a population growing exponentially the growth can be determined as follows:

$N = No \exp(KGT)$

$2N = No \exp(KGTd)$ i.e. the number of cells to double in an elapsed period of time (Td or cell doubling time)

$\ln 2 = KGTd$

$KG = \ln 2/Td$

and the doubling time $Td = \ln 2/KG$ (Steel 1977, Aherne et al., 1977).

The time taken for a tumour cell population to double in size, depends on the median intermitotic time (Tc), the growth fraction (Ip) and the rate of cell loss (KL). Thus the rate of cell loss (KL) may also be derived according to this expression:

$$KL = \ln(1+Ip)/Tc - \ln 2/Td$$

modified after Lala and Patt (1966), Steel,(1968) and Lala,(1971).

Steel and Bensted,(1965) and Steel,(1968, 1977) have described the growth efficiency of a tumour which was not subject to cell loss, but influenced by the growth fraction. The parameter is the potential doubling time (Tpd).

In terms of the cell cycle time (Tc), the doubling time will depend on 'a' as shown by Steel (1977):

$$Td = (\ln 2/\ln a)Tc.$$

The potential doubling time will be equal to the cell cycle time if the growth fraction is unity; and if there were no cell loss, Tpd is equal to tumour doubling time.

In terms of birth rate

$$Tpd = \ln 2/KB = \ln 2\ tm/Im = \lambda\ m\ tm/Im$$

and in terms of the labelling index

$$= \lambda\ ts/LI$$

where λ is a correction factor, which depends on Ip and the position of S in the cell cycle, (Johnson 1961, Lamerton and Steel 1968, Steel 1977). In an exponentially growing cell population in which tm is short compared with the cycle time

$\lambda m = \ln 2 = 0.693$

and λ s will lie between ln2 and 2ln2, depending on the position of S phase within the cell cycle.

The cell loss rate may be expressed in terms of Tpd and Td that is,

$$N = No \exp (\ln 2/Td)T$$

the growth of the population can also be formulated, using KB which is expressed in terms of Tpd:

$$KB = \ln 2/Tpd,$$

and KL the cell loss rate that is influencing the tumour

$$\text{i.e. } N = No \exp (\ln 2/Tpd - KL)T$$

therefore

$$KL = \ln 2/Tpd\ (1-Tpd/Td\ ) = \ln 2/Tpd - \ln 2/Td$$

therefore the ratio of cell loss to the rate of cell birth

$$= KL/KB = [\ln 2\ (Td-Tpd)/\ (Tpd)(Td)]\ [\ Tpd/\ln 2] = 1-Tpd/Td = \phi$$

ϕ = the "cell loss factor" as defined by (Steel 1968, 1977) as loss expressed in terms of a fraction or percentage of total cell birth per unit of time. When Td is large, ϕ approaches a high cell loss value. When Td equals Tpd, ϕ equals zero. Tumour size increases when ϕ is small, but grows relatively slower when ϕ is larger.

In many tumours ϕ is around 0.5 and in some it is around 0.9; Steel (1968, 1977), (Tubiana 1971), (Malaise et al., 1973), (Rajewsky 1974), (Denekamp and Fowler 1977), (Denekamp 1982).

ϕ expresses a size effect: a difference of volumes contributed by cells produced and cells lost. ϕ does not indicate the way in which cells were lost, nor whether they died before removal, or lost as viable cells via the blood stream, lymphatics or exfoliation at a surface.

If dying cells within a tumour progressively contribute to the increase in the mean tumour volume per cell, then the tumour doubling time is a biased estimate of the actual population doubling time within a tumour; i.e. the tumour volume will be increasing faster than the cell population. Therefore, cell loss will be underestimated.

Other factors also contribute to the enlargement of tumour size; when the mean tumour cell volume does not remain constant (the size distribution or cells may change with time); accumulation of intercellular substances and fluids (Steel 1968); lymphocytes and macrophages migrating into the tumour (Elboim et al., 1977, Stern 1983).

Also an increase in the ratio of the tumour stroma relative to tumour cells (Denekamp 1972), contributes as with the above mentioned examples to tumour size, and thus underestimating cell loss. Areas of necrosis present in the tumour at various tumour sizes, when remaining in constant relation to tumour size; the cell loss factor will reflect invariably the actual loss. But if the areas of necrosis were to increase with time as tumour grows, and

contributing to tumour size then, cell loss will be underestimated (Steel 1968) and a correction of ϕ is required, Begg (1977).

KL is important in defining cell loss per unit time from a growing cell population, but it does not reveal the magnitude of this loss on the cell production rate as ϕ does.

In some results, the derived values of KL were not that appreciable, within the range of tumour doubling times, as has been shown by Lala and Patt (1966), and by Steel (1968) on the results of Lala and Patt (1966). This was also indicated in the results of cell loss described by Watson (1976) and Smadja-Joffe (1981).

When Steel (1968) applied the cell loss factor ϕ to the results of Lala and Patt (1966), one can notice an appreciable increase in terms of cell loss in relation to cell birth rate, with increasing volume. This was not due to an increasing KL but due to the expression of cell loss in terms of a decreasing cell production rate.

There is another form of cell loss, which is a programmed form of cell death, or apoptosis (Kerr and Searle 1980).

Apoptosis may be described as a genetically programmed process of cell deletion. The cell shrinks through a mechanism from within the cell, finally breaking up to form "apoptotic bodies". These cell fragments are phagocytosed by adjacent cells and macrophages. Apoptosis, therefore, may have an important role in cell kinetics, as the regulation of population size may not only be viewed in terms of rates of cell production but also in terms of rates of cell deletion.

1.1.7 Tumour growth:

Tumour growth is characterised by a mixture of growth rates. Tumours do not have a single growth rate, but rates which vary with tumour growth. There is a silent interval of growth at the earliest periods of tumour growth, where the new transformed cells that have developed are proliferating and selecting themselves against the host's environment. When the host's environment counteracts the new group of clones of proliferating cells, some tumour cells are killed and some may interact with the host's response in a way which consequently make these new tumour cells dormant. When conditions are favourable, they will grow.

Tumour growth may be expressed or described in terms of various mathematical relations, taking into account tumour size and the period of tumour growth. Exponential growth may be used to describe the growth of tumour cell populations. Growth according to an exponential is characterized by a constant rate of growth (the specific growth rate) in terms of cell number or volume, with time, and is proportional to the number of cells or volume at an instant in time (Steel 1977),(Aherne et al., 1977) .

The cell number would therefore increase or double its size according to the following equation:

$$N = No\ 2^{t/Tc}$$

$$N = No\ e^{mt}$$

where N = number of cells at time t; No = initial number of cells; 2 is the base for the growth equation where each cell produces 2 cells; t = is the time of observation; Tc = median intermitotic

time; m = slope of the curve.

The volume of a tumour may also be expressed as the equation above:

$Vt = Vo \exp \ln 2\ t/Td$

Vt = volume at time t, Vo = initial volume, t = time of observation, Td = tumour doubling time.

and on a logarithmic scale describing the growth curve, the equation is:

$$\ln Vt = (0.693/Td)t + \ln Vo \text{ (Steel 1977)}$$

The growth rate, is equal to $\ln 2/Td$ which is the slope; and Td is the tumour doubling time.

Cell proliferation is not uniform within a tumour, as the tumour contains a mixture of cells with respect to their intermitotic times. The cells contributing to tumour growth retain the growth characteristic from their anscestors, each cell will double its number according to its own cell cycle time.

Tumour growth analysis may assume serial estimation of cell number with time, as in ascites tumour growth in vivo or a cell suspension in vitro. In solid tumours, measurement of tumour volume or weight may be used for tumour growth analysis. Tumour volume may be an approximation when using the following formulae:

$V = \pi/6 \times (\text{mean diameter})^3$, or

$v = [\text{length} \times (\text{width})^2]/2$ (Steel 1977)

or the product of 3 principal diameters as described by Dethlefsen et al., (1968).

The tumour volume or weight can be transformed into logarithimic scale, and growth can be assessed with respect to time. Such growth curves usually show progressive slowing of growth as the tumour enlarges, and the curves usually bend toward the time axis. The plot of the growth curve in general is not an exponential (although the earliest stages may be so) , but often fits a Gompertz equation; i.e. an exponential growth with an exponential retarding process superimposed upon it. The mean diameter of the tumour may also be used in a linear scale against time to assess tumour growth, (Steel 1977).

Mean diameter versus time usually gives a linear relationship when compared to the usual curvature produced on the logarithmic scale of tumour volume versus time, (Denekamp 1982).

Another type of growth curve assessment that gives a linear relationship with time, as compared to the logarithmic scale of volume, weight or cell number, is the cube root function.

The growth of ascites in vivo described by Klein and Revesz (1953) relating the increase of cell numbers on the logarithmic scale to time on the linear scale, followed a smooth convex curve along the time axis. When the increase in cell number was transformed into its cube root value, a linear relationship with time was obtained. The growth of ascites described by Lala and Patt (1966) using the logarithim of the increase in cell numbers versus the linear scale of time, was exponential, at least for the initial period up to 3 days, the curve was bent towards the time

axis at later periods of growth. The growth of the ascites described by Barford and Barford (1980b) was nearly exponential for the earliest period of growth, but eventually curved towards the time axis. Thus to express a relationship for the ascites growth Patt and Blackford (1954) suggested that the initial stages of growth may be approximated by an exponential fit, and later stages by the cube root transform; and that neither fit can describe the overall pattern of the growth curve. The cube root growth has been described by Mendelsohn (1963, 1965), Mendelsohn and Dethlefsen (1968), and Steel (1977). Mendelsohn (1963-1965) summarized the patterns of growth by using the "rate of change of growth" equation

$$dy/dt = ky^b$$

where y is the number of cells or the volume of the tumour, t is time, and k is a growth constant. The mode of growth is defined by the exponent b. When b = 1, the rate of growth is exponential and is proportional to y. When b is zero, rate of growth is linear and is equal K. Values for b intermediate between zero and one, show that the growth rate increases in proportion to y raised to its corresponding power. Of the potential growth rates generated by intermediate values of b is one in which the cube root of y increases linearly with time; and this cube root transform is applicable when $dy/dt = ky^{2/3}$ (Mendelsohn 1963) and this type of growth rate may be applicable if tumour growth rate were influenced by waste products, growth control substances, or diffusion of nutrients through the surface of the tumour. Steel (1977) has suggested that tumours need not be restricted only to this mode of growth when nutrient supply to the tumour is proportional to its surface area; but also by the internal vascular supply being distributed in such a way that there is no uniform central

necrosis, but a patchy distribution of avascular regions. In both cases it may be that tumour growth depends on the total nutrient supply and therefore to the tumour surface area, which results in cube-root growth. The cube-root growth may represent a balance between the tumour's specific growth rate and its rate of growth decay, (Laird 1969). Steel (1977), described various equations for growth such as the logistic and the Gompertz. The Gompertz has been widely used and has been described by Laird (1964, 1966, 1969), McCredie et al., (1965), Norton et al., (1976), Steel (1977, 1980), Aherne et al., (1977), Gratton et al., (1978), and Brunton and Wheldon (1978, 1980).

Tumour cells proliferate by a modified type of an exponential process, in which successive doublings occur at increasingly longer intervals: the specific growth rate for most tumours decreases with time, the decrease in the growth rate is exponential with time. Thus in the Gompertz equation

$$V/Vo = \exp[A/B\ (1-\exp\ (-Bt)]$$

V is tumour volume at any time t, Vo is the initial tumour volume, A is the initial specific growth rate, B is the growth constant that governs the decrease in growth (i.e. that determines the rate of growth increase).

At the earliest period of growth when t is very small, or in the special case when B is equal to zero, then:

$$V = Vo \exp\ (At)$$

and growth is exponential, but apart from the two conditions mentioned above, tumour cells will proliferate by a modified

exponential in which successive doubling will occur at increasingly longer intervals. The tumour reaches its plateau or asymptote according to ratio A/B; and can not pass this maximum value even when time is extended. The number (n) of successive tumour doublings is related to ratio A/B, i.e. $n = A/B \ln 2$. The larger the ratio of A/B the greater the number of doublings.

The retardation in the tumour exists at all times, and the size of volume at time (t) in

$$V = Vo \exp [A/B(1-\exp(-Bt)],$$

is independent of the retardation process; the only factors that bear on whether growth of a tumour is near to or farther away from being simple exponential growth (and then throughout growth) is the ratio of the specific growth (A) to its rate of decay (B), (Laird 1969).

Wheldon (1980) suggested a composite curve, a Gompertz-exponential known as "Gompex" to describe a tumour curve beginning with an exponential type, then shifts to Gompertz at later stages of growth. The tumour has the usual three parameters, i.e. (initial number of cells, the growth and retardation constants) and a fourth parameter, the number of cells at which the transition from the exponential to Gompertz occurs.

There are many factors which contribute to the slowing of tumour growth, such as the progressive development of necrotic areas within the tumour, and these areas may increase as the tumour volume increases. Metabolic by-products and cytotoxic substances of necrosis are harmful to other proliferating cells within the tumour,(Holmberg 1968).

The availability of nutrients and blood supply to the tumour is also a factor that contributes to growth, (Burton 1966), (Summers 1966). Inadequate vasculature, and hence blood supply to the tumour, may also contribute to the retadation of tumour growth, (Tannock 1968, McCredie et al., 1971, Hirst and Denekamp 1979, and Hirst et al., 1982). Apart from the above mentioned factors that influence tumour cell proliferation, Burns (1969) has suggested that homeostatic regulation mechanisms were involved in tumour growth control. Burns,(1968-1969) has suggested that the growth of ascites tumour cells in vivo had stopped because of the existence of a self-inhibition of growth mechanism that depended on the attainment of a critical number of viable tumour cells, mediated via the ascitic fluid. Homeostatic control mechanism may operate on tumours which retain some characteristics of the tissue of origin. Trotter (1961) has shown in the hepatoma of mice (where tumour growth was compared between partially hepatectomized and normal mice), that the time taken for the tumour to appear was shorter in partially hepatectomized mice than in normal mice. This was also shown to occur in some early transplant generations, and not later generation transplants or established hepatomas. Trotter (1961) has also shown that the time taken for tumours to appaer after implantation in mice, was generally, inversely related to the transplant generation number, i.e.: the higher the transplant generation number, the shorter the period for the tumour to appear.

McCredie et al., (1971) have compared the growth rates between a spontaneous C3H mammary tumour and its (900th) transplant generation, and have shown that the growth rate in the transplant generation increased, due to a higher (A/B - Gompertz constants) ratio than that in the spontaneous tumour. In the spontaneous tumour the initial growth rate was slow because the cells were well

differentiated, their generation time was long, while the Gompertz retardation constant (B) was small because of uniform blood supply. In the transplant generation there was a marked decrease in the fraction of tumour cells and an increase in necrosis. Poor differentiation of the tumour cells, have probably accounted for the initial rapid rate of growth, and the decrease in the relative vascular volume for the marked retardation (the B coefficient of the Gompertz equation). Steel et al., (1971) using the rat mammary fibroadenoma compared the changes in the growth rates between the spontaneous primary tumour and its corresponding transplant generations. The pattern of growth in the primary tumour, was irregular and slow, while that of the transplanted tumour was regular, and faster. The growth rate increased as the number of transplant generations increased. Serial transplantation of the tumour, influenced several parameters of the tumour growth. The volume doubling time Td, the median intermeitotic cell cycle time Tc, and the cell loss factor, decreased with serial transplantation, while the growth fraction Ip increased. Similar results were demonstrated by Steel (1972) and Kovacs and Evans (1977). Steel et al., (1971) have also shown that there was a kinetic relationship among the growth rates of the respective transplant generations. The initial growth rate of the fourth transplant generation was similar to the final growth rate of the third transplant generation. The same link existed between the third and the second transplant generations.

Steel (1977) has suggested, that the general rule upon serial transplantation, was an accelerated growth rate. Some exceptions to this rule, has been described by Begg (1971), where the growth rate slowed down upon successive transplantation, together with a change in the histological character of the tumour, and an increase

in Tc (median intermitotic time). In general, therefore, with successive transplantation there is an associated increase in the growth potential of the tumour cells, through cellular selection and adaptation, (Nowell 1976, 1978), (Kovacs and Evans 1977), (Steel and Stephens 1982).

The tumour growth rate also corresponds to the histological type of tumour, (Charbit et al., 1971), (Malaise et al., 1973), (Steel 1977), (Tubiana 1982). Also there are differences in the growth rates among the primary tumours between different anatomical sites; and also between the primary and metastatic growth, (Steel 1977). The growth of the tumour may also be influenced by the immunological environment of the host in which the tumour is growing, (Janik and Steel 1972, Rajewsky and Gruneisen 1972, Riches and Brynmor Thomas 1971).

Tumour cells are not to be assumed as undergoing an uncontrolled process of high frequency cell division contributing to tumour size. The general trend for the length of cell cycle time (median intermitotic time, Tc) in tumour cells is usually shorter than its counterpart in normal cells, (Denekamp and Fowler 1977).

However, Tc, is not necessarily shorter in tumour cells than in some normal cells. Baserga and Wiebel (1969) have shown that in mice, the normal duodenal crypt cells have a Tc of 10.3 hours, whereas a transplanted fibrosarcoma has a Tc of 16.5-17.5 hours. Hasegawa et al., (1976) have shown that in the mouse cervix, the tumour cell cycle time was longer than its normal counterpart. When the tissue tends towards excessive growth, from a normal balanced proliferative state, to hyperplasia and neoplasia, so would Tc tend towards shorter durations, (Bresciani 1968).

The growth of a cell population is regulated by the length of the cell cycle (median intermitotic time), the fractions of dividing and non-dividing cells, and the fraction of G_0 or quiescent cells that may contribute to the population under an appropriate stimulus. An expansion in growth can be achieved by shortening the length of the cell cycle, increasing the growth fraction with recruitment from G_0 and a decrease of cell loss, or any combination of these, (Baserga 1976).

Considering some of the available data on the change of Tc which is associated with tumour growth, (Frindel et al., 1967) have shown a very small increase in Tc with increasing tumour size, in the NCTC fibrosarcoma grown as a solid tumour in mice. Simpson-Herren and LLoyd (1970, as described by Lala 1977) have shown an increase in the adenocarcinoma cell cycle time with tumour growth. Lala (1972,1977) showed a very small increase in cell cycle time in the Ehrlich ascites tumour growing in solid form, with progressive tumour growth. The plasmacytoma described by Simpson-Herren and Lloyd (1970 , as described by Lala 1977) had an increase in the cell cycle time between 6 and 7 days of tumour growth but no significant change in Tc was observed at later tumour growth between days 7 and 19. Several authors have also reported some increase in Tc with increasing tumour size, (Simpson-Herren et al. 1974, Simpson-Herren 1977, Zobl et al., 1975, and Feaux De Lacroix and Lennartz 1981).

Gunduz (1981), on the other hand, showed no correlation between tumour volume and an increase in cell cycle time; there was a slight increase in Tc for a short period, with no increase in Tc for the rest part of tumour growth. Proliferative activity within an individual tumour (solid tumours) also varies from the periphery

to the centre of the tumour. Hermens and Barendsen (1967) observed minimal changes in Tc, although variations in labelling indices existed between periphery and centre. Tannock (1968) observed that the labelling and mitotic indices were declining as the distance between cells and their blood supply increased; the cell cycle time was nearly constant but the growth fraction dropped substantially as the distance increased between the cells and their blood supply. Rockwell and Kallman (1972) observed no change in Tc and a minimal change in Ip between periphery and centre, although Kovacs and Evans (1977) reported some increase in Tc at the tumour centre relative to the periphery. Hirst and Denekamp (1979) studying the relation of vasculature to tumour cell proliferation observed a decreasing LI and Im as the cells were at a further position from blood supply. These decreased indices were associated with an increase in cell cycle time and small changes in Ip.

Hirst et al., (1982) have also observed higher LI and Im indices near blood areas relative to the poor supply areas, and have demonstrated that capillaries were not being formed fast enough to keep with the rate of tumour cell proliferation. The poor blood supply may be responsible for a low growth fraction.

Kovacs and Evans (1977) using the H411E tumour cultured in vitro, observed an increase in Tc with progressive tumour growth. Woo et al., (1980) also reported an increase in the Tc of Burkitt's lymphoma cell cultured in vitro. Similarly, the growth of the ascites in vivo was associated with a marked trend of increasing cell cycle time, with progressive tumour growth as described by Lala and Patt (1966), Frindel et al., (1969), Tannock (1969), Schiffer et al., (1973), Dombernowsky et al., (1973), Schiffer and Markoe (1974), Lala (1977), and Muller et al., (1982).

In ascitic tumours growing intraperitoneally,the deceleration of the growth rate is associated with a marked increase in Tc , a common feature associated with many ascites. The slowing of growth may also be linked to changes in the growth fraction and the amount of cell loss, or any combination of these. Tannock (1969) showed that the increase in Td was due to an increase in cell loss, but a nearly umiform Ip.

Muller et al., (1982) using the JB-1 ascites also observed an increase in Td with increasing tumour age. An increase in Tc was the main cause for an extended Td , as the growth fraction of unity was nearly constant, and cell loss was almost zero up to 5 days of growth.

Several factors may contribute to the inhibition of ascitic tumour cell proliferation with increasing age, such as cell crowding, lack of nutrient and oxygen supply and accumulation of waste products (Tannock 1969). Specific inhibitors of tumour cell proliferation also contribute in controlling tumour growth. Barford (1981) and Barford and Scherbeck (1982) have demonstrated the effects of chalones which were present in the ascitic fluid on the specific inhibition of cell influx from G_1 to S-phase.

In solid tumours the change in cell cycle time is not as marked as that found in the ascites. The growth fraction in some solid tumours decreases as the tumour grows in size, (Frindel et al. 1967), (Watson 1976), (Lala 1977), (Steel 1977), (Feaux De Lacroix and Lennartz 1981), (Gunduz 1981). Some exceptions to a decreasing growth fraction, were reported by Simpson-Herren and Lloyd (1970-as reported by Lala 1977) and Zobl et al., (1975). Steel (1977) and Denekamp (1970, 1982) described the relation between tumour growth, median intermitotic time, growth fraction

and cell loss factor.

Denekamp (1970, 1982) has suggested that there was a correlation between an increasing median intermitotic time (Tc) and an increasing tumour volume doubling time (Td) among sarcomas rather than carcinomas. Steel (1977) showed that there was no correlation between an increase in Tc and an increase in Td among mice tumours, although there was some tendency for increase in the cell cycle time of the sarcomas and carcinomas of rats and hamsters with an increasing Td, including early transplant generations. There was a relatively better correlation between a decreasing Ip and an increasing tumour Td in the sarcomas, than in the carcinomas as described by Denekamp (1970-1982). This was also present in Steel's data (Steel 1977) among the tumours in mice, rats and hamsters, including early transplant generations.

Cell loss contributes an important part in determining the overall growth rate of tumour, and is often the main determinant of a slow growth rate, (Steel 1977). Cell loss is more important in spontaneous tumours than in tumours which have been serially transplanted for many generations, (Steel 1977) and (Denekamp 1982).

Denekamp (1982) has shown that when tumours of similar size were considered, all of which growing as syngeneic transplants, there appeared a correlation between the cell loss factor and tumour volume doubling time (Td), especially with slow growing tumours such as the carcinomas, rather than relatively faster growing sarcomas, in which the main contributor was the median intermitotic cycle time (Tc). Denekamp (1982) has suggested that the cell loss associated with the carcinomas, may reflect the origin of carcinomas from epithelial surfaces where rapid cell

production is balanced by rapid cell loss.

1.2. Cell kinetic techniques:

The kinetic parameters which have been discussed earlier can be estimated by some experimental procedures, and the purpose of this section is to describe the stathmokinetic method and briefly review other methods which are associated with the estimates of cell kinetic parameters.

1.2.1 Metaphase arrest. The stathmokinetic method:

Substances that arrest dividing cells in metaphase (stathmokinetic agents), provide a useful method of studying cell production in tissues, (Wright and Appleton 1980). The first compound to be used in arresting metaphases was colchicine. Colchicine was obtained from the autumn crocus (Colchicum autumnale), (Eigesti and Dustin 1955, Stevens Hooper 1961, Wilson et al., 1974, Aherne et al., 1977). There is a variety of stathmokinetic compounds apart from colchicine, such as colcemid (a derivative of colchicine) (Puck and Steffen 1963, Clarke 1971, Smith et al., 1974, Thorud et al., 1980, and McDermott et al., 1985), and the vinca alkaloids vinblastine and vincristine (Frei et al., 1964, Tannock 1967, Smith et al., 1974, Al-Dewachi et al., 1975, Barford and Barford 1980a,b, Camplejohn et al., 1980, Aherne et al., 1980, Jones and Camplejohn 1983, Samperiz et al., 1985, and Wynford-Thomas 1985).

Vincristine [$C_{46}H_{54}N_4O_{10}$] is obtained from the periwinkle plant, Vinca rosea L. of the family Apocynaceae, (Johnstone et al., 1963), used as vincristine sulphate (Oncovin: produced by Eli Lilly Plc., U.K.) for the treatment of malignant disease.

Vincristine has been shown by Smith et al., (1972-1974) to be a more effective stathmokinetic substance, when compared to vinblastine and colcemid. The stathmokinetic compounds when added in vitro to cultures of proliferating cells or given in vivo to laboratory animals or human patients, causes proliferating cells to accumulate in the metaphase stage of mitosis. The action of these mitotic inhibitory compounds is due to their interaction with tubulin in the cell, which results in the dissolution of microtubules (disruptive action on metaphase spindles), (Taylor 1965 , Wilson et al., 1974 , Aherne et al., 1977 , Thorud et al., 1980) , thus keeping the chromosomes arrested in metaphase, with a characteristic clumped chromatin (short, thick and sometimes fused chromosomes, ball or plate like in appearance (Stevens Hooper 1961, Taylor 1965, Clarke 1971).

Tannock (1967) proposed that an effective stathmokinetic agent should possess the following characteristics:

1. There must be an optimum dose, which is able to arrest all metaphases in the tissue of interest over a defined period of time.

2. The arrested metaphases should not degenerate into an unrecognisable state before the tissues can be examined for the number of metaphases.

3. The mitotic arrest properties should not be highly sensitive to dose.

4. The stathmokinetic substance should have no harmful effects on interphase cells.

Tannock (1967) has stated that if these criteria were satisfied by any agent, it would show a broadly-peaked dose curve and a reasonably well-defined linear collection period in a linearity study. Tannock (1967) compared the relative effectiveness of several stathmokinetic substances on rat intestines and transplantable tumours, and concluded after comparing the kinetic results obtained with the stathmokinetic substance with the results obtained by direct tumour measurement, that vincristine appeared to have the advantage of becoming almost immediately available after injection and furnished the most accurate estimates of both tumour doubling time and the mean cell cycle time of the epithelial cells of the crypt.

When the entry of cells into metaphase is asynchronous, the rate at which metaphases collect after using a stathmokinetic agent, can give an estimate of the rate of cell production. The frequency of arrested metaphases in a cell population in an ideal case, should be in direct proportion to the duration of metaphase arrest, that is, the rate of metaphase collection is independent of the duration of metaphase arrest. Therfore, a linear collection of metaphases is obtained, when the rate of entry into metaphase is constant, and the metaphases must be completely blocked by the stathmokinetic agent and must not degenerate.

The mitotic index Im can be a guide to the rate at which tissues are proliferating, but it is inadequate because Im depends on the rate at which cells enter mitosis (rm), and also on the time period the cells stay in that phase, i.e.: (mitotic duration tm) , (Wright 1975, Aherne et al., 1977, Steel 1977, Thorud et al., 1980).

Therefore: Im = tm.rm

The flux of cells into mitosis depends on the proportion of cycling cells in the whole cell population and on the duration of the average intermitotic (cell cycle) time of the cycling cells i.e. Ip and Tc,

Therefore Im = Ip.tm/Tc for steady state

and Im = (1n1+Ip).tm/Tc for exponetial growth (Wright 1975).

Thus, under optimum conditions the rate of cells accumulating at metaphase, will be equal to rate of entry into mitosis i.e. at mitosis one cell generates two daughter cells, therefore rm is the slope of the curve of the metaphase collection function and is also the birth rate (KB).

Thus in steady state

rm=Im/tm

rm=Imet/ta (1) (Aherne et al., 1977).

Imet, is the metaphase index at the end of the arrest period (ta). Therefore, Imet = ta + tm/Tc (Wright and Appleton 1980) and thus the birth rate can also be calculated in terms of the turn-over time, i.e. : the potential doubling time or apparent cell cycle time.

Thus, KB = rm = 1/Tca , for steady state conditions (Aherne et al., 1977).

In exponential growth, where the frequency of cells immediately after mitosis is higher relative to the number of cells entering mitosis.

Im = exp t/Tc ln2 - 1 (Smith and Dendy 1962)

t is the time at which cell proliferation may be scored for the number of cells in mitosis.

A similar equation to that described by Smith and Dendy (1962) is that described by Puck and Steffen (1963):

Im (mitotic index) at time interval (t) after the addition of colcemid,

$$Im = 2^{tm+t/T-1}$$

tm = mitotic period

t = is the elapsed time after the addition of the stathmokinetic substance

T = the total generation time.

Re-arranging in terms of the base (e)

Im = exp ln2(tm+t)/Tc -1

and re-arranging in terms of the common logarithm (Log_{10}),

$\log_{10}(1+Im)=0.301/Tc(tm+t)$ ta≥tm, assuming a growth fraction of

unity.

$\log_{10}$ (1+Im) which is similar to $\log_{10}$ (1+Imet) is called the "Collection Function" as described by Puck and Steffen(1963) as $\log_{10}$(1+NM), NM being the mitotic index,

0.301/Tc is the slope and 0.301tm/Tc is the intercept, and therefore:

rm = log (1+Imet)/ta : which is similar to equation 1.

and thus:

rm = 0.301/Tca when the growth fraction is unknown,

Thus: KB = 0.301/Tca

An optimal dose of the stathmokinetic agent must be determined for the tissue to be studied, (Wright and Appleton 1980). Tannock (1967) and Nome (1975) have shown that the optimal dosage of different stathmokinetic substances were different in the same tissue, the same stathmokinetic substance has different optimal dosage values in different tissues, as will be described below. Barford and Barford (1980a) have shown that the LIA2 ascites were sensitive to a dose of 0.05ug/ml vincristine, which was the dose that achieved the most effective metaphase accumulation, while for the JB-I-E and PNJ ascites 0.05ug/ml vincristine showed anaphase and telophase escape. Thus as the dose of the stathmokinetic substance increases, so does the number of accumulating metaphases increase, up to a certain limit; as Tannock (1967), Nome (1975), and Duffill et al., (1977), have demonstrated that the number of accumulating metaphases will drop as the concentration of the

stathmokinetic substance increases beyond the optimum dose.

There are other effects to stathmokinetic substances. Fitzgerald and Brehaut (1970) have shown that the rate of DNA synthesis was depressed in response to high doses of colchicine. Similarly, Hell and Cox (1963) have reported that colchicine inhibited DNA synthesis in the guinea pig ear epidermis in vitro, but they have also reported that colcemid was less inhibitory at the same dose. In a different study Riches et al., (1981) have also reported differences of sensitivity by tissues towards vincristine; thus while a certain dose of vincristine did not suppress DNA synthesis in the maternal thymus, this same dose suppressed DNA synthesis in the foetal thymus. Clarke (1971) has concluded from his experimental study on the crypts of Lieberkuhn of the rat, that metaphase-blocking agents (vinblastine and colcemid) at suitable doses do not affect the rate of entry of cells into mitosis. The increase in metaphase numbers per increase in stathmokinetic dosage is due to the arrest of an increasing proportion of metaphases, (Tannock 1967), and as described by Taylor (1965), using colchicine in in vitro cultures of human cells, the effect of increasing the concentration of colchicine was to decrease the interval before metaphase accumulation began. Puck and Steffen (1963) have also observed a lag period in the action of colcemid in arresting metaphases; thus if the rate-limiting process involved colcemid diffusion, the lag should be decreased by increasing the colcemid concentration in the culture medium; however, as Puck and Steffen (1963) have observed, a 20-fold increase in colcemid concentration had no effect in shifting the curve to the Y-axis intercept. This implied that colcemid had been specifically prevented from acting on all cells which have already entered mitosis, even though they have not yet reached the point at

which the blocking action was exerted. Hence, only the cells that were exposed to colcemid before entering mitosis, were accumulated on reaching the metaphase-anaphase region, (Puck and Steffen 1963).

An optimum dose of a stathmokinetic agent to achieve a complete metaphase arrest in terms of a defined collection time period is necessary, to insure against anaphase escape and against metaphase degeneration, because if metaphase arrest was not complete, and blocked metaphases were degenerating due to an unsuitable dosage used, or extending the period of metaphase collection, then the mean birth rate (KB) will be underestimated with the consequence of overestimating the mean apparant cell cycle time (Tca); (Stevens Hooper 1961, Puck et al., 1964, Tannock 1967, Clarke 1971, Aherne and Camplejohn 1972, Wright 1975, Aherne et al., 1977, Wright and Appleton 1980, and Riches et al., 1981). The metaphase arrest technique is a relatively easier method to perform in cell kinetic studies, as compared to other methods which involve the use of tritiated thymidine.

1.2.2 The use of radioactively labelled nucleotides:

3H-Tdr and 125IUDR are specific lables for DNA, and when either of these labelled nucleotides are made available to cell populations, cells in S phase incorporate these labelled nucleotides into DNA. 3H-Tdr incorporation into DNA provides several methods for analyzing cell population kinetics, such as the pulse labelling index, the fraction of labelled mitosis technique (FLM), continuous labelling, double nucleotide labelling and grain counting. The uptake of 3H-Tdr and 125IUDR by DNA can also be assessed by scintillation counting.

1.2.2.1 The labelling index:

Exposure of a cell population to labelled IUDR or Tdr for a short period will enable the proportion of cells in DNA synthesis to be determined. This fraction of labelled cells is therefore called the pulse labelling index (LI). Its use as a guide to the proliferative activity is similar to that of the mitotic index, however the labelling index is a larger fraction than the mitotic index, thus the LI has a relatively smaller error than the Im, (Aherne et al., 1977).

1.2.2.2 Double labelling:

The double labelling technique enables the duration of S phase and the flux rates into and out of S phase to be determined, (Aherne et al., 1977). This technique makes use of the energy difference between the beta particles emitted by 14C and 3H. The underlying principle of this method is that between the first and second applications of labelled thymidine , a certain fraction of cells have left DNA synthesis phase, hence will not be labelled by the second isotope. The fraction of cells leaving the S phase in a given time duration is proportional to the ratio:

length of interval/total duration of S phase

Thus the duration of S phase can be calculated.

1.2.2.3 The FLM technique:

This technique is one of the earliest and informative of the isotope techniques used in the study of the mitotic cycle parameters.

3H-Tdr is made available to the cell population for a period of time, and tissue or cell samples are later removed and fixed at preset time intervals. Histological sections are prepared from the samples and the cells that have taken up the labelled nucleotide can be distinguished from unlabelled cells by the use of autoradiography, (Aherne et al., 1977).

Figure "B" shows an FLM curve of an idealized asynchronous cell population, without variations in the durations of the respective phases. The cohort of 3H-labelled cells can be followed through the mitotic stage (window) in the serial samples by observing the rise and fall of labelled mitotic cells with the passage of time.

The FLM remains zero, for a time interval of the minimum duration of G_2, and the FLM value starts to increase until it fills (100%) the mitotic window, in a time interval equal to tm, and after a certain time interval the mitotic count drops to zero in a time interval equal to ts - tm. The curve, then continues to rise again, after a time interval of $tg_1 + tg_2$.

In real experiments such idealized curves are not found due to the variation in the rates at which individual cells traverse the phases, thus the second peak is somtimes reduced in height, or eventually due to the randomization within the "cell cycle" the FLM curve dampens and flattens.

The duration of the respective intermitotic phases and the intermitotic time (Tc) are usually read from the 0.5 level. This gives approximate median estimates for the cell population under study, (Aherne et al., 1977). The interval between the 3H-Tdr labelling to the 0.5 labelling point on the ascending limb of the

THE FRACTION OF LABELLED MITOSIS CURVES.

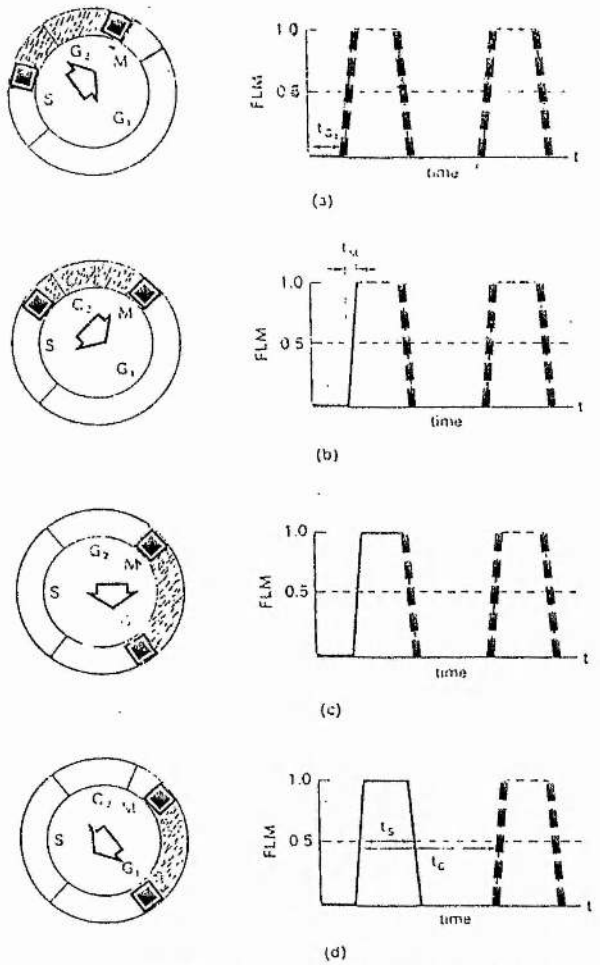


Fig. B: On the left of the diagram a cohort of labelled cells is depicted, moving through the cell cycle. As it moves into M one sees an increasing proportion of labelled mitoses, as shown on the right of the diagram. The time interval from the injection of ^{3}H-thymidine to the median of the ascending limb of the idealized first wave measures $t_{G_2} + (1/2)t_M$. When the cohort of labelled cells has occupied M completely, as at (*b*), the proportion of labelled mitoses is unity (or, as it is sometimes stated, 100 per cent). This is represented on the right by the peak of the idealized first wave. In (*d*) the cohort is moving away from M into G_1. When it reappears the duration of the cycle (t_C) will be measured by the interval from the median of the first wave to the median of the second.

Redrawn from Aherne et al., (1977).

first wave is equal to $tg_2 + 1/2\ tm$. The median S phase period is the interval between the 0.5 labelling points on the ascending and descending limbs of the first wave of the labelled mitosis curve. The time interval of $tg_1 + tg_2 + tm$ is equal to the interval between the 0.5 labelling point on the descending limb of the first wave and the ascending limb of the second. The intermitotic period (Tc) is equal to the interval between the 0.5 labelling points on the first and second ascending mitotic waves. Tg_1 may also be estimated by subtracting $tm + ts + tg_2$ from the median Tc value. When there is no obvious second peak on an FLM curve, the curve describes a plateau level (L). The plateau FLM value depends on the ratio of ts to Tc

Therefore: $L = ts/Tc$

thus $Tc = ts/L$.

Ip can also be calculated after determining LI for the cell population after a pulse of 3H-Tdr;

therefore $Ip = LI/ts/Tc$.

1.2.2.4 Continuous labelling:

In this method, the cell population under study is continuously exposed to 3H-Tdr, so that all cells entering S phase will be labelled. In vivo, the label is made available by continuous infusion, or by repeated injections at periods shorter than the duration of S phase, (Steel, 1997). This technique is best suited to in vitro applications, where the label can be added without perturbing the culture. Samples of the cell population under study, are removed and fixed at intervals of time depending on the nature of the experimental study, and the proportion of

labelled cells is recorded. The labelling index is thus determined at each time interval. If the time period for continuous labelling is extended, all proliferating cells become labelled, thus eventually the LI for the population will reach a plateau value after a time interval which is equal to the mean durations of tg_2+ tm + tg_1. The interval tg_2+ tm is the time required to label all mitosis.

The value of tg_1 is equal to:

$(tg_2 + tm + tg_1) - (tg_2 + tm)$.

The rate of entry into S (labelled cells per unit time) can be estimated, preferably before any labelled cells have had time to divide. The duration of S phase, can therefore be calculated after determining LI:

ts = LI/rs

rs is the slope of the labelling index, (steady state), and in expanding populations

ts = LI (Tca/ln2), (Aherne et al., 1977).

Tc can be estimated from the the combined sum of ts, tg_2 , tm and tg_1.

1.2.2.5 Grain counting:

In this technique, the cell population under study is pulsed with 3H-Tdr as in an FLM experiment, and later, the intensity of labelling on the interphase cell's nucleus is assessed autoradiographically, with the passage of time, (Aherne et al., 1977).

The mean (or median) number of grains per nucleus is usually used to estimate Tc and ts. Thus after labelling with an isotope , cell samples are assessed in terms of a suitable number of nuclei, and a distribution of the grain count is recorded. For the estimate of Tc, it is assumed that the number of grains over an an individual cell is halved at mitosis. Therefore, the halving time for the average (mean or median) grain count is an indication of the duration of the intermitotic cycle.

The first halving time, after the labelling with isotope is less than Tc, as the cells have a delay period of ts and $tg_2 + tm$ to complete the cycle. After the first halving interval, the successive halving intervals should occur at intervals equal to Tc. Thus if the mean grain count is plotted against time, Tc can be estimated if the growth fraction is 1. If some cells decycle after being labelled, a skewed distribution of grain counts will be evident, and the average grain count will decrease more slowly, (i.e. for Ip<1).

This method is not as accurate as the FLM curve, but may be used when mitotic figures are rare and a construction of an FLM is difficult. In continuous labelling experiments , the maximum number of grains over a nucleus occurs when the whole genome has replicated, and this is achieved in a period equal to ts.

1.2.2.6 Scintillation counting:

In this technique, the uptake of isotope by tissues can be assessed in terms of the radiation particles emitted by the tissues. Tissue samples are made soluble in the hydroxide of hyamine 10x, followed by mixing with an organic liquid scintillator, which consists of a flourescent substance and an

aromatic solvent, (Mak and Till 1963, Aherne et al., 1977).

The beta particles emitted by 3H or 14C labelled Tdr, or the gamma particles emitted by 125IUDR strike the flourescent compound, to produce flashes of light which are detected by means of a photomultiplier device present in the scintillating machine. There are advantages in scintillation counting, such as the relative ease of obtaining results as compared to autoradiography. However, when a tissue is composed of different cell types, scintillation counting can not reveal which cells were labelled. Scintillation counting can not distinguish between the changes of isotope uptake, which may be either due to a change in cell number entering S phase (taking up the isotope), or to a change in the amount of isotope taken up by each cell.

In some experiments 125IUDR is the isotope used rather than 3H-Tdr, because 125IUDR is relatively less reutilizable than 3H-Tdr by cell populations and the gamma rays emitted by 125IUDR are relatively easier to detect than the low energy beta particles emitted by 3H-Tdr.

1.2.3 Microdensitometry:

In this technique the quantity of DNA in the nuclei of a cell population is estimated. Tissue fragments are flattened on microscope slides and are stained with a DNA specific stain such the Feulgen. The Feulgen stain varies with the quantity of DNA, and by using an instrument that measures the light absorbed by Feulgen stained nuclei at a specific wave length, one can assess whether cells have 2n DNA (G_1 or G_0), or 4n DNA (G_2 cells). S phase cells can be recognized and excluded by combining the technique with pulse labelling and autoradiography. In a cell

population where all cells are proliferating, the distribution of cells in the respective cell cycle phases can be determined, G_1 and G_2 by densitometry, S by pulse labelling and mitotic cells visually. Thus the age distribution can be known, together with the relative durations of the phases of the cell cycle. There are some difficulties in the assessment of results using this technique, such as the inability to distinguish between G_1 and G_0 cells (tumours), and in cases where the cells contain variable amounts of DNA, (polyploid and anueploid cells).

Microflourometry is another method which estimates the amount of DNA per nucleus in the form of a histogram, and the proportion of cells in G_1, S, G_2 and M can be recorded. In this technique, propidium iodide is the substance used to stain the DNA. The advantages of this method, is the large number of cells (cell suspension) that can be quantified in a short time. However, the method is not suited for the study of structured tissues, where proliferative rates vary with the position in the tissue microarchitecture.

1.3 The regulation of cell proliferation:

The regulation of cell reproduction is an intricate, yet a coordinated process. Cellular proliferation is controlled by many factors, among which are the humoral mediators, such as hormones, chalones, cyclic AMP and polyamines. The effect of these substances on cell proliferation are usually interrelated, as the effect of one mediator may be complemented by the action of another. Lymphokines are also an essential group of substances which contribute to the regulation of proliferation of the immune

system. Most of the early work on lymphokines was associated with the macrophage, and in this regard, the macrophage is also another essential contributor to the regulation of cell proliferation. The contributions of some of the above mentioned mediators to cell proliferation will be discussed below.

1.3.1 Cyclic nucleotides:

When cells reach a high density , intracellular cAMP level rises as compared to low cAMP levels in sparse density cultures (Seifert and Paul 1972). Bannai and Sheppard (1974) have shown that cell contact increases intracellular cAMP levels, reaching peak levels and remaining constant thereafter at the peak level of maximum cell density, when cells were not proliferating. That the initiation of DNA synthesis is associated with a drop in the levels of cAMP was observed when quiescent cells were stimulated to proliferate by adding insulin, (Pastan et al., 1975). Similarly, Parsad (1981) has shown that the replication of certain clones of hepatoma cells were inhibited by cAMP and its analogue dibutyryl-cAMP (Bt_2 cAMP). The association of high levels of cAMP with cellular quiesence, and low cAMP levels in the initiation of DNA synthesis, suggests that cAMP may act as a regulator which may contribute positively or negatively towards the control of cell proliferation (Boynton and Whitfield 1983), (Hunt and Martin 1980). Adenylate cyclase which is present in the cell membrane, catalyses the formation of cAMP. Cyclic AMP acts as a mediator between extra-cellular stimuli and intracellular constituents, and is involved with other factors that contribute to the control of cellular proliferation. The association between hormone effect on target tissue and increases in cAMP levels were demonstrated by Sutherland (Malkinson 1978). Cyclic AMP acts as a second messenger

for hormone action and contributes to various cellular metabolic and physiological functions, and is also involved in the replication process of DNA, (Malkinson 1978, Boynton and Whitfield 1983, Hunt and Martin 1980).

Stimuli by substances which are involved in the control of cell proliferation such as prostaglandins (Samuelsson et al., 1975), chalones (Laurence 1979), lymphokines (Waksman 1979) may mediate their action by increasing intracellular cAMP levels.

The intracellular cAMP levels are also associated with other regulators of cell proliferation, such as the polyamines, as Boynton et al., (1980) have shown that there was a link between increased ornithine decarboxylase activity and surges in cAMP accumulations. The intra-cellular concentration of cAMP induces the formation of a protein kinase which contributes to various physiological functions, through protein phosphorylation.

The cAMP-dependent protein kinase inhibits protein synthesis and polypeptide chain formation (Greengard 1978). The cell flux of mutant cell lines that lack protein kinase activity is not inhibited at the G_1 phase of the cell cycle by Bt_2cAMP, while normal cells which have protein kinase activity respond to Bt_2cAMP and are arrested in the G_1 phase of the cell cycle (Insel et al., 1975). The intra-cellular concentration of cAMP during the cell cycle may show increases at different phases in the cell cycle; G_1, S or G_2, but a common observation was that the nucleotide level at mitosis was lower than other phases (Hunt and Martin 1980). The cyclic nucleotide influences the cell cycle in various ways: (1) increasing or decreasing recruitment of cells from the resting phase G_0 into the cell cycle at G_1, (2) regulating the passage of cells through certain control points within the cycle; (3)

stimulating or inhibiting the entry of cells into G , (Pardee 1974, Pastan et al., 1975, Hunt and Martin 1980).

Boynton and Whitfield (1983), have shown that certain concentrations of cAMP and its derivatives and agents that elevated cAMP levels, arrested cells in G and G phases of the cycle. MacManus and Whitfield (1969) have shown that low concentrations of cAMP stimulated DNA synthesis in rat thymic lymphocytes, although higher concentrations of the cyclic nucleotide inhibited their proliferation. Malamud (1969) also related the effect of isoproterenol on stimulating cell proliferation on the increasing levels of adenylate cyclase activity. Hovi and Vaheri (1973) showed no significant drop in cAMP when quiescent chick fibroblasts were stimulated into cycle by fresh medium. In the salivary gland stimulated by isoproterenol to proliferate, two surges of cAMP were produced, (Tsang et al., 1980-1981), (Boynton and Whitfield 1983), the second smaller cAMP surge was needed for the initiation of DNA synthesis. Similar cAMP surges in kidney cells stimulated to proliferate by folic acid have been reported by Whitfield (1980).

Another cyclic nucleotide, cGMP, like cAMP, may also be involved in the regulation of cell proliferation. Goldberg et al., (1976) have suggested that cGMP and cAMP function together in a yin-yang fashion to control growth and cellular function where the ratio of cGMP to cAMP was important. Seifert and Rudland (1974) have demonstrated a small stimulatory effect on the incorporation of thymidine in serum starved 3T3 cells by cGMP. Rudland et al., (1974) have shown that when cells reached confluent levels in culture, the levels of cGMP decreased while cAMP increased. When the cells were growing before reaching saturation density, cAMP level was low while cGMP level was high. When confluent resting

cells were stimulated to proliferate by the addition of serum, cGMP level increased and cAMP decreased. In other cell culture systems, Hovi et al., (1974) showed that upon the release of chick embroyo fibroblasts to proliferate from density dependent inhibition of growth, there was no significant alteration in the values of cAMP and cGMP. Boynton and Whitfield (1983) have suggested that cAMP was more of a positive regulator than a negative one, because some quiescent cells did not drop their cAMP upon proliferation, and the examples of quiescent cells in the salivary glands, kidney cells and hepatocytes which progress into DNA synthesis with high levels of cAMP.

Raising cAMP levels does not necessarily inhibit cell proliferation but keeping the nucleotide at high levels at certain points in the cycle may influence cell proliferation. Thus Rechler et al., (1977) have shown that when the cAMP level was prevented from falling in human fibroblasts during the first 4 hours after serum stimulation, did not stop the initiation of DNA synthesis, but if this high level was maintained later in G_1 phase, it will prevent initiation into DNA synthesis. Millis et al., (1974) have also shown, that preventing the cAMP level from falling from its G_2 peak prevents the human lymphoid cells from entering mitosis.

Coffino et al., (1975), have demonstrated that exponentially growing lymphoma cells were blocked in G_1 phase, when treated with Bt_2cAMP. The cell cycle of a mutant lymphoma line which lacked the cAMP-dependent protein kinase was not affected by Bt_2cAMP. The mutant lymphoma have been shown to maintain a normal cell cycle with a similar doubling time as their normal lymphoma counterparts. Thus, Coffino et al., (1975) have suggested that cAMP may not be an essential regulator of cell proliferation.

Parsad (1981) described some of the data in the literature on the involvement of cyclic nucleotides in cell transformation, and showed that some carcinomas and fibrosarcomas have low levels of cAMP compared to their normal counterpart tissues. Parsad (1981) has shown that an increase in cAMP levels in certain clones of mouse neuroblastoma cells in culture, induced and increased the expression of biochemical and morphological functions which were characteristic of mature neurons, such as the formation of long neurites, blockade of the cells in G phase of the cell cycle, and an increase in the levels of cAMP phosphodiesterase. Cyclic GMP did not show a significant role in regulating the expression of differentiation in neuroblastoma cells. Thus cAMP can contribute in several ways to the control of cell proliferation.

1.3.2 The Polyamines:

The aliphatic polyamines, putrescine, spermidine, and spermine, are normal cell constituents that contribute an essential function in cell proliferation and differentiation; (Gaugas 1980), (Heby 1981), (Pegg and McCann 1982), (Goynes 1982). Some of the contributions of polyamines to cell proliferation will be discussed below together with a description of polyamine biosynthesis: spermine and spermidine are formed from their precursor putrescine. Putrescine is formed by the direct decarboxylation of L-ornithine. L-ornithine is formed through the hydrolysis of L-arginine by the enzyme arginase.

Polyamine biosynthesis starts with the hydrolysis of L-arginine by L-arginine amidinohydrolase, to produce L-ornithine, CO_2 and urea.

An increase in the activity of arginase and the formation of spermidine from L-arginine has been described by Oka and Perry (1974).

L-ornithine decorboxylase (ODC, L-ornithine carboxy-lyase E.C. 4.1.1.17) catalyses the formation of putrescine from ornithine. L-methionine and ATP acting as substrates for methionine adenosyltransferase E.C. 2.5.1.6.) to produce S-adenosyl-L-methionine. S-adenosyl-L-methionine decarboxylase (SAMD) (E.C. 4.1.1.50) produces decarboxylated S-adenosyl-L-methionine from S-adenosyl- L-methionine (SAM). SAMD is an important enzyme in the synthesis of spermine and spermidine. Spermidine synthase catalyses the transfer of the propylamine group from SAM to putrescine to yield spermidine. Spermine synthase transfers the propylamine group from SAM to spermidine to form spermine, (Scalabrino and Ferioli 1981), (Pegg and McCann 1982). Dykstra and Herbst (1965) were the first to suggest that polyamines might be involved in the proliferative response of cells in regenerating liver, where there was an increase in spermidine in the early period of the regenerative process.

Andersson and Heby (1980) have demonstrated in the Ehrlich ascites tumour cells growing in vivo, that the concentration of spermine and spermidine progressively increased from day 6 after transplantation until day 12 upon reaching stationary phase of growth, after which there was no increase in the levels of polyamines. The levels of RNA and to some extent DNA parallel the increase in polyamine from day 6 to 12. By using inhibitors of polyamine biosynthesis, the requirement and involvement of these

cationic molecules was demonstrated.

Fillingame et al., (1975) have used methylglyoxal bis-gaunylhydrazone or MGBG which inhibited the action of SAMD, to inhibit spermine and spermidine production in Con-A stimulated lymphocytes. In these polyamine deficient cells the rate of thymidine incorporation was inhibited. The MGBG block was reversed by the addition of spermidine or spermine, thus, Fillingame et al., (1975), postulated that the increased levels of spermine and spermidine seen in rapidly proliferating tissues were necessary for enhanced rates of DNA replication. The rates of RNA and protein syntheses have decreased, together with a reduction in DNA content when E-coli mutant (SpeA) was partially blocked in arginine decarboxylase, and thus starved for polyamine. Thus polyamine have some effects on RNA, protein and DNA syntheses (Morris and Harada 1980).

The levels of polyamines have a certain pattern around the cell cycle, and as described by Heby and Andersson (1980), the levels of spermine, spermidine, putrescine and ODC have 2 peaks in the cell cycle of CHO fibroblasts. The synthesis of polyamine was initiated in mid-G_1, and started to accumulate towards late G_1 phase. The rates of syntheses of polyamines, and their content, were highest when the cells started to synthesize DNA. The activity decreased markedly during mid S-phase, and towards the end of S-phase, but started to increase again, that is through G_2 and mitosis, and prior to cell division the polyamine levels were as high as those observed at G_1/S boundary. After cell division the polyamine content decreased.

Thus polyamines are required for the normal progression of these cells during the cell cycle, as Andersson and Heby (1980) have shown that when MGBG was injected in Ehrlich ascites tumour bearing mice, the ascites started to accumulate at the S and G_2 phases.

The process of cell division was also obstructed as Sunkara and Rao (1981) have shown that the process of cytokinesis was affected by the depletion of polyamines. Cells treated with MGBG contained about 40% lower levels of spermidine and spermine, than those from untreated cell cultures. The decrease in polyamine levels was associated with a significant increase in binucleate cells, (Sunkara and Rao 1981). Addition of spermidine or spermine to mitotic cells collected from (MGBG) treated cell cultures reduced the incidence of binucleate cells.

Bethell and Pegg (1981) have found that the conversion of resting 3T3-L1 fibroblasts into adipocytes when stimulated with insulin, was associated with an increase in the intracellular levels of spermidine.

Addition of a-difluoromethyl ornithine (DFMO), depleted cellular polyamine levels and prevented the conversion of fibroblasts into adipocytes. The inhibitory effect of DFMO was reversed by the addition of spermidine and putrescine along with the inhibitor. This suggested that polyamines were needed in the process of differentiation.

Orinithine decarboxylase is an important enzyme in the biosynthesis of polyamines and its activity may be influenced by other humoral mediators of proliferation, such as, Bt_2 cAMP which depresses the activity of ODC (Coffino 1981). The lymphokines also

influence ODC, and this will be discussed later in the lymphokine section.

Apart from the requirement for polyamines in cellular proliferation, their presence in tissue cultures environments that contain FCS or polyamine oxidase, will change their properties, so as to become inhibitory to cellular proliferation. Spermine and spermidine may be available in tissue extracts as free molecules or tightly bound to larger carrier molecules. The thymic chalone described by Allen et al., (1977) and the JB-1 ascites chalone described by Barford (1980), both contained spermine, both were complexed with carrier molecules, and both were inhibitory to their target tissues.

1.3.3 The chalones:

Weiss and Kavanau (1957) sugggested the template-antitemplate theory in which tissues controlled their growth by a negative feedback mechanism. Each cell reproduced its protoplasm by a mechanism in which key compounds (templates) characteristics of the particular cell type acted as a stimulator for growth. Each cell also produced specific free diffusable compounds antagonistic to templates (antitemplates) which can block the reproductive activity of the corresponding templates. Thus when there was abundant antitemplates, blocking the effect of templates, the growth rate of the cell population will decline and eventually there will be a balance between templates and antitemplates and growth stops. If the level of anti-templates decreases due to tissue loss, growth will start again. Osgood (1957) has also suggested that cell proliferation was regulated by humoral inhibitors of cell division.

A substance which can contribute to the control of cell proliferation by exerting a specific inhibitory action on the cellular proliferation of its tissue of origin, has been demonstrated by Bullough and Laurence (1960) in their experiment of wound healing on the mouse ear. Bullough (1962, as described by Bullough 1977 and Laurence 1979) termed such an inhibitory substance a chalone.

The chalone has been defined by Bullough (1962, as described by Laurence 1979) as "an internal secretion produced by a tissue for the purpose of controlling by inhibition the mitotic activity of the cells of that same tissue". The specific inhibition of mitotic activity in the limb blastema by the newt limb chalone was demonstrated by Coomber and Scadding (1983), and the control of population size in spermatagonia was described by de Rooij et al., (1985), where the length of the period of active proliferation of the undifferentiated spermatognia was regulated by a negative feedback from the differentiating spermatogonia.

The characteristics of chalones have been described by Thornley and Laurence (1975), Bullough (1977), Allen and Smith (1979), Laurence (1979), Patt and Houck (1980) and Iversen (1981), along similar lines, and Iversen (1981) has summed up these characteristics:

(1) Chalones are naturally occurring, physiological cell proliferation inhibitory substances, and are water soluble.

(2) Chalones are tissue or cell line specific or show tissue or cell line preference or selectivity.

(3) Chalones must be produced in and be present in the tissues on which they selectively act. They are probably mainly produced by the more mature cells of the series.

(4) chalone action is non toxic and reversible, therefore chalones do not injure cells or cell membranes.

A variety of chalones of various tissues of origin have been reported in the literature as described by Laurence (1979) and Iversen (1981). Chalones are proteins or polypeptides with or without carbohydrate moieties, with a range of molecular weights from 500 to 50000, (Thornley and Laurence 1975), (Allen and Smith 1979) and (Patt and Houck 1980). Iversen (1981) has suggested that there was a possibility that chalones in vivo were composite molecules consisting of a small peptide molecule which was the general growth inhibitor (which need not be tissue specific) coupled to a tissue specific glycoprotein which was specific for each type of differentiated cell. Chalones modulate the length of the cell cycle. Chalones block the entry of cells in to S phase and mitosis as has been shown in the epidermal G_1, and G_2 chalones (Laurence et al., 1979), and as shown by Elgjo et al., (1981) the cell flux in the epidermis was inhibited at G_1 -S, S-G_2 and G_2 -M transitions. Lord (1975- as described by Laurence 1979) has shown that the granulocytic chalone blocked the flux of cells form G_1 to S phase, together with an increase in the cell cycle time. Chalones may also accelerate the maturation rate of granulocytes as has been described by Benestad and Rytömaa (1977).

The action of chalones is effected through the cell membrane. The association between the effects of chalone, adrenaline and cyclic AMP was first demonstrated by Bullough and Laurence (1964), which led Iversen (1969) to suggest a possible role for cAMP in

chalone effects. Houck and Attalah (1975- as described by Laurence 1979) have suggested that the chalone was part of the cell membrane which activated cAMP via adenyl cyclase, and that the chalone maintained a high level of adenyl cyclase activity (Attalah and Houck 1976). Laurence (1979) has suggested with regard to experimental evidence on chalone action on the epidermis, that cell proliferation may be raised either because chalone was depleted or cAMP was lowered or both. The activity of ornithine decarboxylase and the levels of spermine and spermidine have not been considered in relation to the effects of chalones as these substances are involved in cell proliferation, and hence, they may be involved somewhere in the sequence of cell proliferation control mechanisms by chalones. Nakai (1976) has shown that the Ehrlich ascites chalone may control the mitotic activity of the Ehrlich ascites by inhibiting alpha and beta polymerase activities in DNA. Furthermore, Nakai and Gregely (1980) have demonstrated that the Ehrlich ascites chalone could inhibit, DNA polymerase alpha, directly within the nucleus without an intermediate step such as a cell membrane receptor. Other factors which may be involved in the chalone mechanism are the antichalones "stimulators" of cell proliferation, as described by Rytömaa (1976) and Laurence (1979). Tumour cells are responsive to chalone action, as demonstrated by Bullough and Laurence (1970), on the L5178Y- mouse lymphoma cultured in vitro, and in which the mitotic activity was inhibited by lymphocyte and lymphoma extracts, which required the co-operation of adrenalin and hydrocortisone to produce this inhibition. The specific inhibitory effects of chalones were also demonstrated by Bichel and Barford (1977). The JB-1 ascites chalone decreased the proportion of JB-1 ascites tumour cells in S phase, while it had no inhibitory effect on the L1a ascites tumour.

Similarly, Barford (1981) has demonstrated the specific and reversible inhibition of influx of the JB-1 ascites tumour cells from G_1 to S phase, with JB-1 ascites chalone preparation. The inhibitory effects of chalones may also be due to polyamines, as Barford (1980) has demonstrated the presence of spermine in his ascites chalone preparation. Tumours may fail to respond to their chalone, probably because the tumour cells may synthesize a small amount of chalone but fail to retain it. Bullough (1977) and Laurence (1979) have suggested that the tumour cell membrane may be altered (i.e. the chalone was leaking into the blood circulation, or the receptors on the outer cell surface were abnormal, thus being unable to interact with the chalone, Bullough 1977). However, tumour cell proliferation can be controlled, if the tumour environment was made "hyperchalonic", (Laurence 1979). Cellular proliferation can be modulated by other substances apart from chalones, such as the interferons which can inhibit or stimulate cell proliferation.

1.3.4 Lymphokines:

Lymphokines are lymphocyte glycoproteins (Pick et al., 1979), that possess hormone activity, secretion by one cell, specific interaction with another target cell, and physiologic regulation under feed back control of specific function, (Evans 1983). Lymphokines maintain homeostasis of the immune system and have the potential to influence the development of cancer, (Evans 1982-1983). Some common Lymphokines are: thymosin, LAF (lymphocyte activating factor), lymphotoxin, MAF (macrophage activity factor), MMIF (macrophage migration inhibitory factor), TCGF (T cell growth factor) and leukocyte aIFN (interferon) and

immune IFN, (Brouty-Boyé 1980), (Evans 1983).

From the above mentioned lymphokines, lymphotoxin and interferon will be described in terms of their effects on cell growth. Interferon was first discovered by Isaacs and Lindenmann (1957) as an antiviral substance present in the supernatant from cultures of chick chorioallantoic membranes which were exposed to heat inactivated virus. The interferons are a heterogeneous family of glycoproteins: type 1 (alpha and beta) interferons are produced by leukocytes and fibroblasts induced by virus infection, while type 2 or immune interferon (gamma interferon) is produced by stimulated lymphocytes by specific antigens or mitogens. Interferons have been shown to have a variety of effects, such as modifying the structure of cell membranes, contributing to cell differentiation, inducing the synthesis of new proteins, and inhibiting cellular proliferation, (Gresser 1977, Brouty-Boyé 1980, Evans 1983, Trinchieri and Perussia 1985). Cells of the immune system such as macrophages, T, B, and NK cells are influenced by interferon, and hence interferon among other lymphokines has an immunoregulatory role. Interferon induces the appearance of new surface markers (MHC class II) in a variety of cell types such as lymphoid cells, mast cells, melanocytes and melanoma cells, and other tumour-derived cell lines (Wong et al., 1983). Conditioned medium from mitogen-induced lymphocytes contain factors, besides interferon gamma that act synergistically with this type of interferon to induce differentiation in immature myeloid cells, and hence their ability to mediate an antibody-dependent cytotoxicity (AD-CMC) against their target cells which were sensitized with antibody, (Trinchieri and Perussia 1985).

Interferon gamma has marked effects on macrophage dependent tumour cell killing (Meltzer et al., 1983), (Pace et al., 1983) and may act as a macrophage activating factor (MAF), as it induces or enhances the cytotoxicity of human monocytes against tumour targets (Prensky 1983). Interferon also contributes to the expression of surface markers associated with cell helper function and antigen presentation, (Steeg et al., 1982), (King and Jones 1983), (Zlotnik et al., 1983). Nakamura et al., (1984) have demonstrated that gamma interferon enhanced the level of antibody response in vivo induced by several antigens, when interferon was administrated at the same time of immunization. Nakamura et al., (1984) have suggested that the effects of interferon may be mediated through the macrophages in terms of antigen processing or presentation. B cells are also affected by interferons, as Sonnenfeld et al., (1978) have demonstrated the potentiating effect of interferons on immunoglobulin secretion when added late during an immune response.

NK cells produce and respond to interferons by becoming more cytotoxic (Senik et al., 1980),and by recruiting pre-NK cells to differentiate them into cytotoxic forms, (Friedman and Vogel 1983). Similarly the interferons produced by T cells may contribute to the generation of cytotoxic T cells (Farrar et al., 1981), (Friedman and Vogel 1983). Interferons inhibit the multiplication of both normal and tumour cells of different species, (Friedman and Vogel 1983). Interferon inhibited the regeneration of liver in partially hepatectomized mice (Frayssinet et al., 1973), and when adminstered daily to suckling mice, interferon retarded their growth and development (Gresser et al., 1975). Interferon gamma has been demonstrated by Broxmeyer et al., (1983) to inhibit the proliferation and differentiation of haemopoietic precursor cells. The proliferation of tumour cells and their growth rates in in

vitro cultures were retarded by interferon and in some comparative studies malignant cells were more sensitive to interferon than non-tumour cells (Gresser and Tovey 1978), (Brouty-Boye' 1980). Interferons are not cytotoxic, and their action is reversible, but if interferons were maintained in the culture medium for more than 48 hours cell death was often observed, (Gresser and Tovey 1978). Interferon interacts with the target's cell membrane, (Auget 1980), inducing the synthesis of RNA and polypeptides related to antiviral proliferation,(Friedman and Vogel 1983). Interferon binds to the cell surface, and a direct correlation has been found between the intracellular cAMP content and the degree of inhibition of cell multiplication induced by interferon, (Fuse and Kuwata 1978). The inhibitory effects of interferon on cell proliferation, may be similar to cAMP, as both induce the formation of protein kinase which contributes to the control of cell proliferation (Greengard 1978).

Collyn d'Hooghe et al., (1977) have demonstrated a marked increase in the generation time of mouse mammary tumour cultures incubated with interferons. Balkwill and Taylor-Papadimitriou (1978) have demonstrated that interferons reduced the rate of entry of Balb/c 3T3 cells into S phase, together with an increase in the duration of G_1 and G_2 + S phases. Creasey et al., (1980) have demonstrated that human leukocyte interferon decreased the transition rate of G_0-G_1 quiescent human melanoma cutures, into S phase, and also prolonged the duration of S phase, as compared to the cultures cultured without interferon.

Some of the events associated with the stimulation of quiescent cells into DNA synthesis was an increase in the levels polyamine biosynthesis enzymes. The induction of the activities of

the polyamine associated enzymes, such as S-adenosyl-L-methionine decarboxylase and ornithine decarboxylase, were inhibited by interferon, (Lee and Sreevalsan 1981, as described by Friedman and Vogel 1983) and (Sreevalsan et al., 1979, 1980). DNA synthesis is also influenced by interferon, as Lundblad and Lundgren (1982) have demonstrated that the treatment of human glioma cultures with interferon-beta, has decreased the activity of DNA polymerase of these exponentially growing cells, and has also decreased their growth rate. The inhibition of cell proliferation by interferon was accompanied by a reduction in DNA, RNA, and protein synthesis, (Brouty-Boyé 1980).

Trying et al., (1982) have shown that interferon gamma was cytotoxic for some cell types. The inhibitory effects of interferons preparations may be due to contamination with lymphotoxins (Stone-Wolff et al., 1984, as described by Trinchieri and Perussia 1985). Most of the growth-inhibiting effects of interferon preparations were not observed when either lymphotoxin free, natural interferon gamma preparation or homogeneous recombinant interferon gamma were used. Interferon gamma had a strong synergistic effect with lymphotoxin, and cells were lysed or their growth was prevented by a combination of interferon gamma and lymphotoxin at concentrations that were inefficient separately, (Trinchieri and Perussia 1985).

The term lymphotoxin was introduced by Granger and Kolb (1968) to describe the cytotoxic effects in cell cultures of soluble products produced by mitogen or antigen stimulated lymphocytes. Lymphotoxin has a variety of effects, such as inhibiting tumour growth in vivo, the induction of increased susceptibility of tumour cells to cytolytic destruction by NK cells, and to prevent the

carcinogen-induced morphological transformation during carcinogensis. Lymphotoxins are glycoproteins, which bind to the receptors on target cells, and may affect the proliferative properties of the target cell, through alterations in the chemical structure of the target cell's membrane (Walker et al., 1976, Kobayashi et al., 1979). The cytotoxic activities of lymphotoxin were demonstrated in terms of the enumeration of surviving cells, isotope release by damaged cells, and colony inhibition assays (Evans 1982).

Lymphotoxin can induce or stimulate interferon secretion (Evans 1983), and thus can augment the inhibitory activities of interferon by its mutual synergistic effect with interferon in inhibiting the clonal growth of HeLa cells as has been shown by Trinchieri and Perussia (1985), and as described earlier, the enhancing effects of interferon on NK cytotoxic activities and recruitment of pre-NK cells into the cytolytic forms. Thus lymphotoxin was controling tumour proliferation in this example in an indirect way.

Moreover, lymphotoxin can increase the sensitivity of tumour cells to NK cell destruction (Ransom and Evans 1982). The anti-carcinogenic effects of lymphotoxin on the transformation of hamster cells by chemical carcinogen or ultraviolet irradiation were demonstrated by Evans and co-workers (Evans 1982).

The anticarcinogenic effects of lymphotoxin are due to its influence on the cell membrane, thus it induces a specific increase in glucosamine incorporation in nontransformed hamster cells, while it decreases the incorporation of glucosamine in transformed hamster cells (Evans 1982).

Lymphotoxin, can therefore, contribute in a variety of ways to the control of tumour cell proliferation; and if its activity is down-regulated, it may be one of the means that a developing tumour cell can escape from the inherent homeostatic mechanisms that control tumour cell growth (Evans 1983).

1.3.5 The macrophage:

The control of cell proliferation, as has been reviewed earlier, was discussed in terms of humoral factors which were involved in cellular proliferation. However, there are other mechanisms in which cellular proliferation may be controlled through cellular co-operation, besides the contribution of humoral mediators. Cell to cell contact is a mechanism of cellular co-operation in which cell proliferation is controlled, (Burger and Noonan 1970, Bunge et al., 1979). Cell to cell contact and co-operation is a feature associated with the cells of the immune system, and one such cell, which contributes an essential role in these activities is the macrophage. The macrophage, practically, handles most of the mechanisms involved in the control of cell proliferation, as they secrete humoral mediators involved in the regulation of cell proliferation, they contribute to the regulation of the immune response as accessory or effector cells, and modulate tumour growth. Cells of the mononuclear phagocytes series originate in the bone marrow. Their differentiation pathway is from stem cells to monoblasts, promonocytes and monocytes; the monocytes are transported by the blood to function in the various tissues as tissue macrophages, and thus the mononuclear phagocytes

are hetergeneous in nature, (Carr and Daems 1980). The supply of macrophages to the peripheral tissue (under steady state physiological conditions) is from the bone marrow (Spector 1977), and (Keller 1979) has demonstrated that the selective radiation damage to the bone marrow by radioactive strontium, has abolished the spontaneous cytotoxity expressed by mononuclear phagocytes from the peritoneum and the spleen. Under conditions of demand, in inflammatory sites, the macrophages increase their numbers by mitotic division (Spector 1977). The differentiation, and survival of mononuclear phagocytes depends on a line-specific growth factor which is selectively destroyed by the differentiated cells (Tushinski et al., 1982).

The various roles of the macrophage such as the control of cell proliferation, mechanisms of natural cytotoxity and release of humoral mediators, and enhancement or suppression of tumour growth will be discussed below. The interaction between macrophages and other cells may result in enhancement or suppression of the various functions of the target cells, in in vitro culures, depending on the functional activity of the effectors and the ratio of target to effector cells. The effector cell may affect the proliferation of the target cell, its viability, differentiation and its functional ability . The growth of cells in in vitro cultures is promoted and regulated by the presence of macrophages or adherent cells (Zipori 1980). Blood monocytes and tissue macrophages produce (CSA) colony stimulating activity substances (Johnson and Burgess 1978), (Cline et al., 1974) which can contribute to the growth and differentiation of stem cells into macrophages and granulocyte colonies in invitro cultures (Polverini et al., 1977), (Gospodarowicz and Moran 1976), (Metcalf 1977-1979). The macrophages contribute a dual role in the regulation of

monocytopoiesis and thus regulating their own numbers, by promoting their growth and survival with colony stimulating factor (CSF) (Tushinski 1982), and limiting their growth by prostaglandins (PGE2), (Kurland et al., 1978, 1979). Macrophage products are also involved in the regulation of erythropoiesis (Kurland et al., 1980), in the promotion of B cell differentiation into antibody secreting cells, in the promotion the immature thymocytes to mature T cells, (Unanue 1978) and in the control of fibroblast proliferation (Leibovich 1978). Macrophages may modulate their target through the production of lymphokines, such as interleukin I (Il1) which is a polypeptide with a molecular weight of 12000-16000 daltons (Oppenheim and Gery 1982).

IL1 augments thymocyte proliferation (Oppenheim et al., 1982) and induces helper activity in thymocytes (Farrar and Koopman 1979). Interleukin I augments immunoglobulin production and influences the lymphocyte membrane by increasing its ability to bind antigen , and by inducing the formation of differentiation markers, such as the stable sheep erythocyte rosette in human lymphocytes (Oppenheim and Gery 1982). Interleukin 1 is also required in promoting the production of interleukin 2 (T cell growth factor) which promotes the proliferation of T cells (Smith and Ruscetti 1981). The macrophages participate as accessory cells in antigen processing and presentation (cell to cell contact) to B and T cells. T-helper cells must interact with macrophages in the process of surveillance for foreign antigens, and then must interact with B cells and cytotoxic (TC) precursor cells to stimulate their differentiation into plasma cells and Tc effector cells respectively, (Hood et al., 1978). The macrophage can participate in a variety of ways to control tumour growth, the simplest form which does not require co-operation with T cells or

their regulatory lymphokines, is by direct natural cytotoxicity, without even the requirement of previous exposure to target cells, as Mantovani et al., (1980) have demonstrated the effective growth-inhibitory activities of human mononuclear phagocytes obtained from various anatomical sites against target TU5 tumour cells in invitro cultures. In another form of target cell interaction, the normal macrophages exposed to tumour cells or antigens either acquired cytotoxicity, or induced cytotoxic lymphocytes. Robinson and Wheelock (1981) have demonstrated that tumour dormancy of a syngenic L5178Y lymphoma ascites tumour in DBA/2 mice was accompanied by cytotoxic T cells in the early phase, but at 50 to 70 days post tumour inoculation, the cytotoxic cells had disappeared but cytotoxic macrophages appeared and were considered to be the mediators of the tumour dormant state. Taniyama and Holden (1979) have demonstrated that the co-operation between spleen cells and syngeneic macrophages was required for the induction of cytotoxicity against their target syngeneic tumour cells. Earlier results by Evans and Alexander (1970) have demonstrated the specificity of the macrophage cytotoxic response. Macrophages obtained from mice challenged with L51787 lymphoma cells, were specifically cytotoxic against the L51787 cells and not other related lymphoma target cells in invitro cultures. Evans and Alexander (1972) have also demonstrated that T cells sensitized with C57BL/6 lymphoma cells, confer the specificity of target recognition to macrophages through a T-cell product, however, after the specific target killing by macrophages, the macrophages become activated and can destroy other targets non-specifically. Macrophages are also involved with specific antibody in antibody-dependent cell cytotoxicity (ADCC) in the destruction of tumour target cells (Bast et al., 1980). Macrophages obtained from

regressing tumours were shown by Russell and McIntosh (1977) to be more cytotoxic than those derived from progressing tumours. Macrophages infiltrate tumours growing in vivo, and reach a fixed percentage in relation to total cellular content, which appears to be specific for each tumour. The level of macrophages in the tumour may decline over many passages in vivo, (Evans 1972). The relation between macrophage content in tumours and tumour immunogenicity was suggested by Evans (1972), and was investigated by Eccles and Alexander (1974) who showed a putative relationship between tumour immunogenicity, tumour macrophage content, and tumour metastatic potential.

In a further study by Evans and Lawler (1980) on mice sarcomas, the relationship between tumour immunogenicity and its macrophage content was tested, and the data indicated a lack of correlation between immunogenicity and macrophage content. Macrophages resident within a tumour are not homogeneous with respect to tumour cell interaction, as Evans and Haskill (1983) have reported the heterogeneous nature of macrophages from different rodent tumours, or within the same tumour, in terms of their sedimentation velocity distributions and their respective effector functions in colony inhibition and ADCC assays. The growth of the tumour despite the presence of the macrophages does not necessarily mean that the macrophages were not active within the tumour, as Key and Haskill (1981) have demonstrated that macrophages from regressing tumours were highly phagocytic and mediated the ADCC against their target tumour cells. The control of tumour cell proliferation, in terms of macrophage tumour cell interaction may be mediated through other mechanisms which may contribute to both tumour growth stimulation and inhibition. Macrophages release a variety of humoural mediators such as

thymidine (Optiz 1975a, b) which interferes with the incorporation of isotope uptake by cell cultures, and which was found to inhibit the growth of EL-4 leukemic cells (Stadecker et al., 1977). The results described by Vercammen-Grandjean and Lejeune (1977) on the inhibitory effects of macrophage supernatants on the uptake of isotope by target cells suggested that the inhibitory effects of the macrophage supernatants can be attributed to factors other than thymidine. Activated macrophages secrete polyamine oxidase (Allison 1978), which reacts with spermine or spermidine to generate inhibitory products on cellular proliferation. Actively dividing cells contain spermine and spermidine, and thus, the availability of polyamine oxidase in the environment of such cells may inhibit or regulate their proliferation. The effects of polyamines and polyamine oxidase on cellular proliferation will be discussed later in this thesis.

Chen et al., (1977) have shown that a factor prepared from thioglycolate induced peritoneal macrophages of DBA/2 mice inhibited the proliferation of L1210 lymphoma cells, and in a subsequent study by Chen and Broom (1980) the inhibitory substance was identified as arginase; the effects of arginase were reversed upon the addition of arginine to the cell cultures. Macrophages produce tumour necrosis factor (TNF), (Pennica et al., 1984) which retards tumour growth, and also produce Cachectin (Beutler et al., 1985) which posesses potent TNF activity in vitro.

Macrophages secrete interferon and prostaglandins, and tumour cells also produce prostaglandins, the prostaglandins provide a mechanism against the macrophage's role in inhibiting tumour growth (Schultz 1980). Cultures of murine peritonal macrophages activated with mitogen, secreted prostaglandins and were not cytotoxic

against their mastocytoma targets. Addition of indomethacin promoted the cytolytic activity of the macrophages, while the addition of prostaglandin prevented it, and thus these mechanisms of macrophage tumour cell interaction were taken by Taffet and Russell (1981) to explain the lack of cytotoxicity of intratumoural macrophages. Suppressor macrophages also contribute to the promotion of tumour growth. Kolb et al., (1977) have demonstrated that the depressed ability of spleen cells from mice bearing plasmacytoma to mount an antibody response was attributable to suppressor macrophages. Kennard and Zolla-Pazner (1980) have demonstrated that normal peritoneal macrophages may become suppressor macrophages for antibody production, when exposed in vitro to factors released by plasmacytoma cells. The inhibitory activity would be eventually exerted through the production of a second factor (the plasmacytoma induced macrophage substance) by suppessor macrophages. Ehrlich et al., (1980) have demonstrated that splenic NK activity was depressed in Balb/c mice bearing a (DMBA) dimethylbenzanthracene induced mammary carcinoma, but that depressed NK activity was re-potentiated when splenic macrophages were removed. Similarly, Gerson et al., (1981) have demonstrated that macrophages infiltrating MSV-induced tumours can actively suppress the cytolytic effector phase of NK cells.

Ting and Rodrigues (1980a,b) have suggested that macrophages may become suppressive in response to some signal from tumour cells and have demonstrated the existance of an immunoregulatory circuit among macrophage subsets for T-cell mediated cytotoxicity. Under the influence of tumour cells, the T-cell function was positively or negatively regulated, through the immunoregulatory circuit of spleen and peritoneal macrophages. Suppressor macrophages may also influence some functions of T lymphocyte which may contribute to

tumour growth control. The termination of the tumour dormant state of lymphoma ascites in DBA/2 mice was mediated by peritoneal suppressor macrophages which inhibited the generation of cytolytic T cells (Robinson et al., 1983). Garrigues et al., (1981) have demonstrated that splenic macrophages from mice bearing MCA-induced transplantable sarcomas did not affect the cytotoxic ability of differentiated T lymphocytes, but they were inhibitory for the generation of cytotoxic T lymphocytes in an MLC reaction. Suppressor macrophages are also present in normal individuals, and their suppressive effects on several immune responses in invitro assays have been reviewed by Varesio (1983). The suppressive effects of macrophages, included the inhibition of mitogen-induced proliferation of syngeneic spleen cells, the inhibition of the generation of cytotoxic T cells in MLR cultures, the inhibition of development of primary antibody response to red blood cells, and the inhibition of maintenance of NK cell activity. These macrophage associated suppressive effects, may suggest a physiological mechanism, through which the macrophage can contribute to the homeostatic control of the immune system (Varesio 1983). Apart from the promotion of tumour growth by suppressor macrophages, the effectiveness of the cytolytic activity of cytotoxic macrophages against their tumour cell targets is restricted to those tumour cells at G_1 -S transition. Thus the cytolytic macrophage which has a limited period of activity against a proliferating cell target would therefore exert little antitumour effect (Evans and Haskill 1983). The macrophage can also produce factors which stimulate the proliferation of tumour cells. Evans (1979) has demonstrated that macrophages from rodent sarcomas were able to "condition" culture medium, the active material with a molecular weight less than 10,000 stimulated the proliferation of

normal, and neoplastic cells.

CHAPTER TWO

MATERIALS AND METHODS

1. Tissue Culture:

Sterile techniques were used throughout tissue culture experiments. All sterile tissue culture procedures were performed in a laminar flow bench which was swabbed with 70% ethyl alcohol before use.

The medium used for tissue culture was RPMI 1640 (Flow Labs. U.K.) The medium was prepared in 100 ml aliquots that were buffered with 20m.moles Hepes (Sigma. U.K.) pH 7.5 and supplemented with 200m.moles L-glutamine (Flow Labs. U.K.) together with benzyl penicillin 50 units/ml and streptomycin sulphate 50ug/ml (Flow Labs. U.K.). Foetal calf serum (FCS) (Flow Labs. U.K.) was heat inactivated at 56 ^{0}C for 30 minutes in a Grant water bath, (Grant Instruments. U.K.).

All instruments and containers used for tissue culture experiments were sterile and were flamed when necessary.

All short term cultures were incubated in a Leec incubator at 37 ^{0}C which was supplied with 5% CO_2. The animals used (mice-rats) were of 7-10 weeks of age. The animals were swabbed with 70% ethyl alcohol before dissection and were placed under a clean perspex hood which was previously swabbed with 70% alcohol.

2. Preparation of cell suspensions:

Preparation of plasmacytoma cell suspension for tumour passaging:

The subcutaneous tumour present in a Balb/c mouse was excised from the mouse and placed in a sterile petri dish and transferred to a laminar flow bench, where it was divided into approximately 3-5 mm pieces. The necrotic tissue was left aside while the (pink coloured) viable portions were transferred to sterile 6 ml bijous (Sterilin 54001-129A. Sterilin. U.K.), that contained 4 ml of 37 ^{0}C prewarmed RPMI medium.

A tumour cell suspension was prepared by the careful disruption of the tumour fragments through the repeated withdrawal and release of the fragments by a 1 ml sterile syringe (Beckton Dickson. Ireland). The bijous were left over ice for one or two minutes for the debris to settle. The supernate was then transferred to 10 ml sterile conical base tubes (Sterilin 61044-144A5. Sterilin. U.K.), and were centrifuged in a refrigerated 4 ^{0}C (MSE.FISON-Chilspin, MSE. U.K.) bench centrifuge for 10 minutes at 1-1.4x10^{3} RPM . The supernate was discarded, while the cell pellet was resuspended in RPMI to the required concentration; usually 5 x 10^{6} cell/ml. The viability of the cells was assessed by Trypan blue exclusion, (Trypan blue: Sigma T0887. Sigma. U.K.). A stock preparation of 0.2% was used at a final concentration of 1:4 with cell suspension, in an improved Neubauer haemocytometer (Hawkesley ltd. U.K.).

The proportion of viable tumour cells in a tumour cell suspension ranged from 45%-75% , mainly due to the incorporation of necrotic material while dissecting the tissue.

Preparation of plasmacytoma cell suspension for tissue culture:

A plasmacytoma tumour was excised and a cell suspension was prepared as previously described. The cell suspension was filtered through a sterile stainless steel sieve mesh.

The clear suspension was transferred to 10 ml sterile conical base tubes (Sterilin. U.K.) and was centrifuged in a refrigerated (MSE. Chilspin) centrifuge for 10 minutes at $1.4x10^3$ RPM.

The supernate was discarded and the cell pellet was resuspended in cold 0.83% NH_4Cl (BDH Chemicals.U.K.)(2 vol. NH_4Cl: 1 vol. cell pellet) supplemented with Hepes pH 7.4 solution at a final concentration of 20m.moles. The cells were left in an ice bath for 5 to 8 minutes, after which the NH_4Cl cell suspension was diluted with generous amounts of cold RPMI, (Boyle 1968). The cell suspension was centrifuged for 10 minutes at $1.4x10^3$ RPM. The supernate was discarded and the cell pellet resuspended in cold RPMI to the required cell concentration after assessing the cell viability with Trypan blue.

Preparation of normal thymocytes suspension for tissue culture:

Mice or rats (7-10 weeks of age) were killed as decribed previously; the thymus was carefully removed with a pair of forceps, and transferred to a sterile bijou containing prewarmed 37 ^{0}C RPMI 1640.

The thymus was divided into small pieces approximately 2-4mm in size and a cell suspension was prepared as described earlier for plasmacytoma cells.

Preparation of CBA mammary adenocarcinoma cell suspension:

CBA Mice were killed as described previously and their mammary tumours were excised as described for the plasmacytoma procedure. The tumour was divided into approximately 3-5mm pieces. The tumour pieces were incubated with collagenase (Sigma. U.K.) , 0.25% in Earle's Basal salt solution Ca^{2+},Mg^{2+} free containing EDTA 10^{-3} molar, in sterile bijous at 37 C. The bijous were rolled on a tube roller for 10-15 minutes. Tryspin (Sigma. U.K.) was added later to the collagenase tumour suspension, at a final concentration of 0.25% and incubated for a further 10-15 minutes on the tube roller. After the tryspin digestion, calf or horse serum was added at a final concentration of 10% per bijou content of tumour enzyme mixture. The final step was to add Deoxyribonuclease (DNASE.Sigma. U.K.) to the enzyme tumour mixture at a final concentration of not less than 10ug/ml of tumour digest, and in some instances the amount of DNASE was double that amount. The cell suspension obtained from the bijous was diluted with prewarmed 37 ^{0}C medium and the contents were transferred into 10 ml sterile tubes and centrifuged for 10 minutes at 1.4 x 10^{3} RPM in a refrigerated bench centrifuge. The supernate was discarded and the cell pellet resuspended in fresh RPMI.

Short term culture: Microtitre tissue culture plates:

Sterilin microtitre trays (U-well type-57102-M24ARTL) were used for tissue culture experiments. Viable cells of certain numbers were dispensed into the culture wells by a micropipette (Finnpipette), together with the substance to be tested, and a radioisotope; the cell dose was replicated in 5 wells.

3. Isotope labels to monitor DNA synthesis :

5-[125I]Iodo-2-deoxyuridine, (sp. activity 5ci/mg. Amersham International, Plc. U.K.) was the main isotope used in tissue culture experiments while [methyl-3H]Thymidine (sp. activity 5 ci/m.mole, 1 ci/ ml. Amersham.U.K.) was used in some experiments.

A sterile stock solution of 10uci/ml of 125IUDR was prepared and 0.2uci (20ul/well) were dispensed by means of a micropipette into each culture well. The same procedure was applied to 3H-Thymidine. Tissue culture wells containing the cell suspension, the assay substance, and the isotope, were placed in a 5% CO_2 incubator at 37 ^{0}C for different periods of time. After the relevant incubation period, the cells were harvested from the wells on to fibre glass filter paper by means of a cell harvester. The Titertrek cell harvester (Flow Labs), operates by delivering PBS buffer (BDH Chemicals.U.K.) to the wells, and withdrawing the contents of the microtitre wells simultaneously, to deposit them on a fibre glass filter paper, (Flow Labs.).

The filters were left to dry for 15 minutes in a drying oven. The portions of the filter that contained the cell deposit of each well; were punched into small plastic tubes; and the DNA uptake of 125 IUDR was assessed in a gamma spectrometer (LKB Instruments.U.K.).

In the case of 3H-Thymidine the filter discs were dispensed into appropriate scintillating vials. The vials contained 5 mls of liquid scintillation in Toluene (BDH Chemicals. U.K.) The scintillation liquid contained (2-5-Diphenyl-oxazole:PPO) 6g/litre and 1.4-Di-2-(5-Phenyl- oxazolyl) benzene POPOP, 0.2g/litre (BDH Chemicals U.K.). The vials and their contents were counted in an SL 30 scintillation counter (Intertechnique. U.K.), using the thymidine setting for a minimum threshold of 25.5 cpm (counts per

minute) with a maximum counting time of 10 minutes per sample. The counts were assessed for quenching due to pellet, and various cell doses were prepared to assess self absorption.

4. Preparation of various sera :

Human serum :

15-20 ml of blood were withdrawn from human volunteers by venipuncture using a sterile 20 ml disposable syringe (Beckton Dickson. Ireland) with a sterile needle (gauge 25G5/8)

The blood was transferred into sterile 10 ml tubes (Sterilin 144AS). The tubes were capped and left to stand for at least 90 minutes at room temperature for the blood to clot. Subsequently the tubes were kept at 4 ^{0}C for 15 minutes to allow the clot to retract. The serum was aspirated and the tubes were centrifuged in a refrigerated bench centrifuge for 10 minutes at 1.4 x 10^{3} RPM. The remaining serum supernate was aspirated. The serum was pooled and aliquoted in sterile bijous and stored at -20 ^{0}C.

CBA and CD1 mice sera:

CBA and CD1 mice were anaesthetized as described earlier, and blood from the respective group of mice was obtained by cardiac-puncture using a sterile 2 ml syringe (Beckton Dickson. Ireland) fitted with a sterile needle (Gauge 23G1. Beckton Dickson. Ireland). The blood of each group was dispensed into separate sterile tubes (Sterilin 144AS) and the procedure for serum collection was the same as described earlier for human serum.

Rat serum :

Serum from rats was obtained according to the procedure described for mice.

Preparation of bovine serum albumin and human serum albumin :

A 4.3% BSA solution, was prepared by dissolving 430mg of bovine serum albumin (Sigma A7030. Sigma. U.K.) in 10 ml of RPMI. The solution was sterilised by passing through a sterile 0.22um millipore filter (Millipore. U.K.). A 4.6% solution of human serum albumin (Sigma A8763) was prepared by dissolving 467.5 mg human serum albumin (Sigma. U.K.) in 10 ml of RPMI. The solution was sterilised by passing through a sterile 0.22um millipore filter. Both BSA and HSA were stored at 4 ^{0}C. The concentrations of BSA and HSA were according to the values given by Altman and Dittmer (1971).

5. Preparation of hydroxyurea, thymidine and polyamine solutions :

A stock solution of 0.1 moles hydroxyurea (Sigma. U.K.) was prepared by dissolving 76.1mg in 10ml of RPMI 1640 and was sterilised through a sterile 0.22um millipore filter. The stock solution was serially diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} moles, for experimental assays.

Preparation of thymidine solution:

A stock solution of 0.002 moles thymidine (Sigma. U.K.) was prepared and sterilised through 0.22um millipore filter. The stock solution was stored at -20 ^{0}C in sterile bijous for further use.

Preparation of spermidine and spermine stock concentrations :

Spermidine (N-[3-Aminopropyl]-1,4 butanediamine) Trihydrochloride , (Sigma S2501. Sigma. U.K.).

Spermine (N,N -bis[3Aminopropyl]-1,4 butanediamine) tetrahydrochloride, (Sigma 2876. Sigma. U.K.).

Spermidine was prepared at an initial stock concentration of 80mg/litre = 0.314m.moles , and was sterilised through a sterile 0.22um millipore filter, and stored in sterile glass bijous at -20 ^{0}C .

Spermine was prepared at an initial stock concentration of 80mg/litre = 0.229m.moles; prepared and stored as for spermidine.

The stock concentrations of spermine and spemidine were diluted respectively to the following ug/culture well concentrations:

8,4,2,0.8,0.4,0.08 and 0.04.

The percentage inhibition of polymaines was calculated as follows:

A=Isotope uptake by cultures without polyamine (control mean counts per minute)

B=Isotope uptake by cultures with polyamine (mean counts per minutes)

C= % inhibition of isotope uptake = [A-B/A] x 100.

The estimate of ID50 (dose of polyamine per culture that produces a 50% inhibition of isotope uptake by cultures) was estimated graphically from the linear portion of respective curves that described the inhibitory effects of polyamines.

6. Preparation of acid insoluble DNA precipitate from plasmacytoma cells :

A suspension of plasmacytoma cells was prepared as described earlier, and was divided into five groups according to the content of FCS-HI per culture group. Group 1: 5% FCS, Group 2: 10% FCS, Group 3: 15% FCS, Group 4: 25% FCS. The plasmacytoma suspension, was dispensed by means of a micropipette into sterile tubes (Nunc. U.K.) at a concentration of 0.5×10^6 cells per tube (200ul per tube). To each culture 0.2uci of 125IUDR were added. The tubes were capped and incubated for 4 hours at 37 ^{0}C in a Leec incubator. Each group contained 5 tubes. Following the incubation, the tubes were spun in a bench centrifuge at 1.4×10^3 RPM for 10 minutes. The supernate was discarded and the cell pellet of each tube was resuspended in 2 ml of 1 normal NaOH solution (BDH Chemicals; U.k.). The tubes were incubated at 37 ^{0}C for 1 hour, and on several occasions, during the incubation period the tubes were vibrated by means of a vibrating machine to facilitate the DNA extraction procedure. To each part of NaOH tissue homogenate, 2.5 parts of a

freshly prepared 10% trichloroacetic acid were added (TCA.BDH Chemicals; U.K.). TCA and the NaOH tissue homogenate were mixed in sterilin tubes (Sterilin 61304 RT30); and centrifuged at $2x10^3$ RPM in a bench centrifuge for 10 minutes. The supernate was carefully removed with a pasteur pipette drawn to a capillary tip (100um in size). The remaining residue was washed with 5% TCA; centrifuged and the supernate removed as described above. The tubes and their contents of the remaining DNA extract were transferred to a gamma spectrometer to assess isotope uptake by DNA. The DNA extraction procedure was according to the method of Bonting and Jones (1957).

7. Preparation of uptake inhibitory factor (UIF) :

UIF was prepared according to the method of Bonmassar et al., (1978). UIF was prepared from rat thymuses and spleens, also from mice thymuses, spleens, lymph nodes, and bone marrow. All RPMI 1640 media for UIF preparation contained benzyl penicillin 50Iu/ml, streptomycin sulphate 50 ug/ml and 50 ug/ml of Kanamycin (Glaxo. U.K.) to prevent microbial and mycoplasma contamination. The rats and mice were between 7-10 weeks of age.

Thymus UIF :

UIF was prepared from rats and mice, each being independent of the other. UIF preparations from mice were never mixed with UIF preparation from rats. Suspensions of thymocytes were prepared as decribed previously.

The final cell suspension was made at a concentration of 1 x 10^7 cells/ml RPMI without FCS. Cell viability as assessed by Trypan blue exclusion was >98%. 5ml of 1 x 10^7 cells/ml suspension were

dispensed into tissue culture grade, 50mm. diameter, petri dishes (Sterilin 57002-302). The tissue culture petri dishes were transferred into clean, 70% ethyl alcohol swabbed incubation boxes. The box had 2 holes in its lid to allow gas exchange. The box and its contents of petri dishes were incubated for 16 hours at 37 °C, 5% CO_2 in a Leec incubator. Following the incubation, the supernatant from the petri dishes was transferred to 10 ml sterile (Sterilin 144AS) tubes and centrifuged in a refrigerated bench centrifuge for 10 minutes at 2100 RPM,(414xg). The supernate from the tubes was collected, pooled and sterilised through a sterile 0.22um millipore filter; collected in sterile universals and stored at -20 °C.

Spleen UIF :

Mouse and rat spleen UIF was prepared in a similar procedure to that of thymus UIF.

Two fractions of spleen UIF were prepared:

A) Whole spleen UIF (RWC= red and white cells, whole spleen fraction UIF).

B) WCF.UIF: spleen cells UIF, except the erythocytes were lysed, and the remaining fraction was the white cell fraction (WCF).(The white cell fraction UIF is also called STD.UIF or standard UIF).

The 5M UIF (five million cells), was prepared as for WCF UIF but at half culture density, i.e. 5×10^6 cells/ml.

Preparation of mouse bone marrow UIF :

Balb/c mice were killed as described earlier, swabbed with 70% ethyl alcohol; their femurs collected and transferred into sterile bijous containing prewarmed 37 ^{0}C RPMI 1640. The femurs were taken out from the bijous with a sterile forceps; and a sterile needle (Yale microlance 01101-11, gauge 25/5) which was attached to a 1 ml sterile syringe (Plastipak Syringes. Becton Dickson. Ireland) containing RPMI was inserted through one end of the femur. Medium was flushed into the cavity, and the contents of the washing were collected from the opposite side of the femur into sterile bijous. The process was repeated until no more particulate matter came from the femur. The crude bone marrow suspension, so collected, was dispersed into a finer suspension by the same needle and syringe, by gently withdrawing the contents of the crude marrow suspension into the syringe and releasing them back into the bijou. This process was repeated several times. The suspension was centrifuged in a refrigerated (MSE. FISON) bench centrifuge for 10 minutes at 1.4 x 10^{3} RPM; the supernatant discarded and the cell pellet resuspended in cold RPMI 1640 to a concentration of 1 x 10^{7} cell/ml. The cell viability as assessed by Trypan blue was >98%.

Lymph node UIF :

Balb/c mice were killed as described earlier and their lymph nodes from the inguinal, axillary and mesentric regions were collected into sterile bijous containing prewarmed 37 ^{0}C RPMI. The nodes were gently teased by carefully withdrawing and releasing the nodes through a 1 ml sterile syringe. The preparation of the final cell concentration for UIF production was described earlier for the previous UIF preparations.

Preparation of freeze-thawed extracts of spleen cells (white cells fraction, Freeeze-Thaw UIF) :

A suspension of rat spleen cells (white cells fraction) was prepared as decribed previously and adjusted to a concentration of 2×10^7 cells/ml according to the method of Bonmassar et al., (1978). 20mls of 2×10^7 cells/ml were dispensed into sterile universals and kept at -20 °C until the suspension was completely frozen. The frozen suspension was thawed completely at 37 °C in a water bath. The thawed suspension was left at -20 °C until frozen, and re-thawed again. The procedure of freeze-thawing was repeated 3 times and the resultant supernatant was assessed for cell count and viability; which manifested neither. The supernatant was transferred into 10 ml sterile tubes and centrifuged in a refrigerated bench centrifuge for 15 minutes at 414xg. The supernate was collected and sterilised through a sterile 0.22um millipore filter. The supernate was pooled and stored in sterile bijous at -20 °C.

Preparation of adherent and nonadherent cells UIF :

Rat spleen suspension (white cells fraction at a concentration of 1×10^7 cells /ml) was prepared as described previously. The supsension was diluted 1 fold to 5×10^6 cells/ml. 5ml of 5×10^6 cells/ml suspension were dispensed into sterile 50mm petri dishes. The petri dishes were transferred into an incubation box and incubated at 37 °C, 5% CO_2 for two hours in a Leec incubator. The preparation of adherent and nonadherent cell fractions was generally similar to the procedure described by Mosier (1967). Following the 2 hour incubation the supernate containing the nonadherent cells was collected into sterile universals (Sterilin

U.K.). The petri dishes were washed gently several times to remove further weakly adherent cells. The washing procedure consisted of adding 2ml of pre-warmed 37 °C RPMI to each petri dish, and following the addition of warm RPMI medium, the dishes were rotated gently by hand. The pooled supernate was centrifuged at 1.4 x 10^3 RPM for 10 minutes in a refrigerated bench centrifuge. The cell pellet of the nonadherent cells was resuspended to a concentration that was adjusted as to the original concentration of 1 x 10^7 cells/ml, and 5ml of which, were dispensed into 50mm sterile petri dishes.

Preparation of adherent cells :

2ml of prewarmed 37 °C Earle's Basal salt solution (Ca^{2+}, Mg^{2+} free) containing 0.25% Trypsin, (Sigma T 8003), and EDTA, (Sigma-EDS) at a concentration of 10 moles , were dispensed into each petri dish. The dishes were rotated gently on an axial shaker at 37 C for approximately 10 minutes. Immediately after trypsinization 500 ul of calf serum were added to each petri dish to stop the action of trypsin. The contents of the petri dishes were pooled and diluted with additional amounts of medium. The contents were transferred into sterile universals and centrifuged for 10 minutes at 1.4 x 10^3 RPM in a refrigerated bench centrifuge. The supernate was discarded and the cell pellet was resuspended in RPMI. The cell suspension was adjusted to a concentration of 1 x 10^7 cells/ml and 5ml containing the adherent cell fraction were dispensed into sterile petri dishes . Several dishes were prepared for the adherent and nonadherent groups. The culture dishes were transferred into incubation boxes for 16 hours at 37 °C, 5% CO_2 in a Leec incubator. A UIF preparation was made of each fraction as described previously. For both fractions a cell loss of 15.8% was

accounted for after centrifugation, before preparing the UIF suspension. The adherent cells were removed by mild trypsinization which was a final resort to remove them from the petri dishes. Although it would have been better to remove them by scrapping the dishes with a spatula tip that was covered with fine rubber tubing coat. The fraction of nonadherent and adherent cells of the total spleen cell (without red blood cells) was 60.91% and 23.26% repectively.

Preparation of macrophage-free spleen cell suspension (Macrophage Free UIF):

The procedure for preparing a macrophage free cell suspension, was according to the method described by Lee, (1980). The carbonyl iron powder was washed and aliquoted into bottles and sterilised on the same day of the experiment for separating cells according to their adhering ability.

10gm of 99+% pure carbonyl iron powder <60um (Gaf.U.K.) were washed with 100ml of 0.85% saline 4 times to remove any toxic material. A U-shaped iron magnet was used to hold the iron particles down in the beaker while pouring off the saline wash. The iron powder was resuspended in 50ml physiological saline (0.2gm iron/ml saline) and was kept in suspension by constant mixing.

3ml of the iron suspension were pipetted into 100 ml sterile screw-cap bottles. The bottles and their iron contents were sterilised by autoclaving at 18lb/in^2 for 20 minutes. Following the autoclaving, the bottles were immediately tapped and shaken to disperse the iron; discourging it to form clumps. If clumps were to be formed, then, an alternative procedure, to avoid iron

clumpping through autoclaving, was to sterilise the iron powder by extensive washing with sterile saline containing penicillin and streptomycin, before dispensing the 3ml aliquots.

A rat spleen suspension (white cells fraction) was prepared as described previously at a concentration of 10^7 cells/ml. The saline contents of the bottles were aspirated by means of a sterile pasteur pipette while the iron powder was held down by a magnet. Each bottle received 8ml of the spleen suspension (8×10^7 cells/bottle). The bottles were capped tightly and incubated in a water bath for 45 minutes at 37 °C. During the incubation period, the bottles were shaken with just enough force to suspend the iron powder completely, every 5 minutes. At the end of the incubation period, the nonadherent cell suspension was pooled from all bottles in a single sterile beaker by placing a magnet under each bottle to retain the iron particles and the adhering cells with it. A magnet was placed for a few minutes under the container, containing the pooled nonadhering cell suspension to allow residual iron powder to settle. Following the settling of the residual iron powder, the cell suspension was transferred into sterile universals and centrifuged in a refrigerated bench centrifuge at 1.4×10^3 RPM for 10 minutes. The cell pellet was resuspended in RPMI 1640 medium without FCS to prepare a macrophage free UIF. The macrophage free suspension was adjusted to the original concentration of 10^7 cells/ml taking into account a 34.5% loss due to the centrifugation steps. 5 ml of the final preparation of the nonadherent cell suspension was dispensed into sterile 50 mm tissue culture petri dishes to produce a macrophage free UIF. The procedure for UIF preparation was described earlier.

Preparation of different molecular fractions of STD.UIF :

Rat STD.UIF was prepared as described previously. The UIF was dispensed into an Amicon apparatus which fractionates solutions according to the molecular separating properties of the Amicon filter. Four types of Amicon Diaflo ultrfiltration membranes (Amicon. U.K.) were used as follows:

1. A membrane that retains substances of 5×10^4 molecular weight and sizes above 5×10^4 . Code: Amicon (XM)

2. A membrane that retains substances of 1×10^4 molecular weight and sizes above 1×10^4 Code: Amicon (PM)

3. A membrane that retains substances of 1×10^3 molecular weight and sizes above 1×10^3 Code:Amicon (UM2)

4. A membrane that retains substances of 5×10^2 molecular weight and sizes above 5×10^2 Code: Amicon (UM05)

10 ml aliquots of STD.UIF (20 ml. total volume) were dispensed into an Amicon Diaflo ultrafiltration apparatus which had a membrane of the 5×10^4 molecular weight type. The STD.UIF was forced through the membrane under pressure [20/in^2.]. The STD.UIF that passed through the filter was collected in a clean beaker and was refractioned on a 1×10^4 molecular weight exclusion filter. The fractioned UIF, was fractioned on a 1×10^3 molecular weight Amicon exclusion filter. The resulting UIF was again refractioned on a 5×10^2 molecular weight exclusion filter, and the UIF which passed through the filter was retained and coded as B500 or below 5×10^2 molecular weight substances.

All the respective filters (1), (2), (3) and (4) were washed in distilled water. The distilled water washings of the Amicon filters were vacuum dried in a drying machine [Edwards-U.K.] and each was resuspended in 20ml RPMI 1640. All the fractions of the UIF were sterilized through a 0.22um millipore filter. The fractionation of the UIF was carried out at 4 ^{0}C.

The STD.UIF fractions are coded as follows:

5 x 10^4 for molecules of 5 x 10^4 molecular weight and above.

10^4 for molecules below 5 x 10^4 to 10^4 molecular weight.

10^3 for molecules below 10^4 to 10^3 molecular weight.

A500 for molecules below 10^3 to 500 molecular weight.

B500 for molecules that passed through the 500 molecular weight exclusion filter.

All the UIF preparations were stored in sterile universals at -20 ^{0}C for further use.

STD.UIF was heated for half an hour at 56 ^{0}C: A sterile universal containing 20ml of STD.UIF was kept for half an hour in a water bath at 56 ^{0}C.

Preparation of dialysed Rat WCF.UIF :

A visking dialysis tubing (size 24/32. Dept., of Biochemistry, Univ. of St. Andrews.) which retains substances of 10^4 molecular weight and above was soaked in distilled water for 1 hour in a clean beaker. The dialysis tubing was transferred into a larger beaker containing double distilled water (DDW) and was heated until boiling. The dialysis tubing was boiled for 3

minutes. The dialysis tubing was later washed in DDW several times. Two knots were tied at one end of the dialysis tubing, and using a sterile 20ml syringe (Beckton Dickson. Ireland), 20ml of Rat WCF.UIF, (STD.UIF) were dispensed into the tubing. Two knots were tied at the top side of the tubing. The UIF was dialysed against 200ml of RPMI 1640 containing Hepes buffer for 3 days at 4 ^{0}C. The buffer was changed twice daily (for fresh buffer) and was continuously stirred with a magnetic stirrer. The UIF was later taken out of the dialysis bag and its volume recorded. The dialysed UIF (which was of 1 x 10^{4} molecular weight and above) was sterilised through 0.22um sterile millipore filter and was stored in sterile universals at -20 ^{0}C for further use.

Each cell culture in microtitre culture wells, which was incubated with any type of UIF, received 0.2 uci 125IUDR, and was replicated 5 times. The assessment of isotope uptake by cultures was described previously.

Preparation of BC3H-1 muscle cell UIF :

BC3H-1 muscle cells were grown in tissue culture grade flasks (Sterilin 311) as monolayers in Dulbecco's medium (Flow Labs. U.K.) pH 7.4; containing 10% FCS, benzyl penicillin 50IU/ml and streptomycin sulphate 50ug/ml. Each flask contained 15ml of medium, that received an inoculum of 3 x 10^{4} BC3H-1 cells (3 x 10^{4} cells/15ml/flask).The cells grew as monolayers; reaching confluence level between 4-5 days. The exhausted medium was aspirated from the flasks. 10ml of prewarmed 37 ^{0}C Earle's Basal solution Ca^{2+}, Mg^{2+} Free, containing EDTA at a final concentration of 10^{-3} molar, and 300ul of the enzyme viocase (Gibco-U.K.) were delivered into the flasks. The flasks were firmly capped and were shaken mildly at 37

°C for at least 20 minutes, on a flask shaker. A sample of the flasks were examined under an inverted microscope (Olympus-Japan) to asses the effect of viocase on separating the monolayer, into a single cell suspension. When that was nearly achieved, each flask received 1ml of calf serum to stop the action of viocase. The cell suspension in the flasks was pipetted up and down vigorously to breakdown cell clumps.

The cell suspension from the flasks was pooled and centrifuged in a refrigerated bench centrifuge for 10 minutes at 1.4×10^3 RPM. The supernate was discarded and the cell pellet was resuspended in RPMI 1640 at a concentration of 1×10^7 cells/ml for the preparation of the (BC3H-1) UIF. The UIF preparation procedure was described earlier. The cell line was a kind gift from Dr J. Aiton (Derpartment of Physiology. University of St Andrews) which was given by Dr J. Patrick (Salk Institute. San Dieg, California, U.S.A.).

8. Preparation of cytospin smears :

Aliquots of cell suspension (100-300ul) were dispensed into cytospin buckets (Shandon Cytospin-U.K.). Glass slides were attached to the cytospin buckets. The slide side facing the bucket was covered with a filter paper that had a hole, which allowed the suspension to pass from the bucket to the slide upon spinning. The cytospin was used at 6×10^2 RPM at room temperature. Cell suspensions that contained no serum had serum added to them (10% final concentration) before cytospinning.

9. Procedure for assessing the optimum concentration of vincristine, required for metaphase arrest for plasmacytoma cultures in vitro :

1mg of vincristine sulphate was diluted to 0.2mg/ml as described in the procedure for metaphase arrest experiments in vivo. The stock concentration of 200ug/ml was diluted to the required dilutions as follows:

1. 35ul of stock vincristine per 7ml of RPMI 1640 medium = 1ug per ml.

2. 91ul of stock vincristine per 7 ml of RPMI 1640 medium = 2.6ug per ml.

3. 280 ul of stock vincristine per 7ml of RPMI 1640 medium = 8ug per ml

4. Plasmacytoma suspension containing 20% FCS-HI.

0.5ml of each of the above concentrations of vincristine were added to 0.5ml of the plasmacytoma suspension, to give a final dilution of 0.5ug, 1.3ug and 4ug/ml respectively in 10% FCS.

10. Procedure for the assessment of the effects of UIF and vincristine sulphate on the metaphase accumulation of plasmacytoma cultures in vitro :

A plasmacytoma suspension (in RPMI 1640. 10% FCS HI.) was prepared as described previously and was aliquoted equally as follows:

1. Replicate samples of 1ml plasmacytoma suspension (0.5ml suspension + 0.5ml RPMI 1640 diluent at 20% FCS HI), incubated for 4 hours at 37 °C 5% CO_2, in a Leec incubator.

2. Replicate samples of 1ml plasmacytoma suspension, containing a fresh preparation of 0.5ug of vincristine sulphate (0.5ug/ml), incubated at 37 °C, 5% CO_2 for its optimum period to collect a maximum number of metaphases (2 hours), as described previously. [500ul cell suspension + 350ul medium + 100ul FCS +50ul oncovin (0.5ug oncovin/50ul in 10% FCS RPMI].

3. Replicate samples of plasmacytoma suspension (950ul) containing UIF at a final dilution of 20%, were incubated for 4 hours. Following the 4 hour incubation period, 50ul of diluted vincristine sulphate (0.5ug/50ul 10% FCS-RPMI 1640) were added to each tube, and were incubated for 2 hours. [0.5ml cell suspension + 200ul medium + 100ul FCS + 200 ul UIF], which included the 50 ul of diluted vincristine.

4. Replicate samples of 1ml plasmacytoma suspension containing UIF at a final dilution of 20%, were incubated at 37 °C 5% CO_2 for 4 hours.

Following the incubation period, replicate samples from each group were transferred into a cytospin centrifuge to produce cytospin preparations. The cytospin preparations were stained according to the Jenner-Giemsa Procedure. The cytospin slides were later examined under a microscope, (10 x 40 = 400 x magnification) to determine the number of metaphases. At least 2000 cells per slide were screened for metaphases.

11. Procedure for determining the labelling index in cell cultures:

The isotope used was [methyl-3H] Thymidine, [sp. activity 5ci/m. mole., 1mci/ml]; and was diluted in RPMI 1640 to yield a concentration of 1uci/50ul. A plasmacytoma suspension was prepared as described previously and divided into 3 groups: A, B, and C.

A: Plasmacytoma suspension in RPMI 1640 without FCS

B: Plasmacytoma suspension in RPMI 1640 10% FCS-HI

C: Plasmacytoma suspension in RPMI 1640 10% FCS-HI and 20% UIF.

Each suspension group was replicated in 5 sterile tubes (Sterilin-142AS)

Group A: The tubes received 500ul of plasmacytoma suspension + 450 RPMI

Group B: The tubes received 500ul of plasmacytoma suspension + 355ul (RPMI 1640) medium + 95ul FCS

Group C: The tubes received 500ul of a plasmacytoma suspension + [165ul (RPMI 1640) + 95ul FCS] + 190ul UIF

All tubes in groups A, B and C were incubated in a Leec incubator at 37 °C 5% CO_2 at the same time, each tube received 1uci of 3H-TDR in 50ul of RPMI. Following the 4 hour isotope pulsing, aliquots from each tube were spun for 5 minutes in a Shandon (Shandon-U.K.) electronic cytospin, at 6 x 10^2 RPM to produce cytospin smears as described earlier. The cytospin slides were left to dry in air for 5 minutes, followed by methanol fixation for 10 minutes, to prepare the smears for autoradiography according to

the procedure of Kopriwa and Leblond (1962). Following the methanol fixation, the slides were coated with (G5) Ilford emulsion and kept in a lightproof dry box, at 4 ^{0}C for an exposure period of 24 hours. Following the exposure, the slides were developed with (T19) high-contrast developer for 2 minutes, and followed by fixation in (Hypo) sodium thiosulphate. The slides were washed after the fixation procedure and stained in Jenner-Giemsa. Following the staining procedure, the slides were dehydrated in several concentrations of alcohol and mounted in euparal.

1. The slides of each group were later viewed at (10 x 40) 400 x magnification and the number of labelled cells determined (a minimum of 3000 cells per slide were counted) and the proportion of labelled cells among unlabelled cells, [the Labelling Index (LI)] was determined and corrected to labelled cell per 1000 cells.

2. The cytospin preparations of each group were examined at a magnification of (10 x 100 oil immersion) 1000 x magnification, and the number of grains (3H-Tdr-grains) per cell was determined, the microscope eye piece had an E9A graticule (Graticules Ltd., Tonbridge, Kent) fixed to it. The area of the E9A at 1000 x magnification was 1.111 x 10 um^{2}. The E9A graticule contains 10 small squares of equal size, the optimum number of grains that could be counted within 1 small square was 50. Thus if a small square was full of grains, and the number of grains was difficult to determine, the small square was assumed to contain more than 50 grains. The E9A graticule was aligned on a labelled cell and the number of grains counted. 100 cells per cytospin slide were observed, and the number of grains/cell was determined. The mean background grain count was estimated by observing 15 random E9A fields , on areas that contained no cells and on cytoplasmic areas.

Nuclei with 4 grains and less were assumed as background count and were not included in the grain count. The grain count was distributed into 3 groups and the number of cells belonging to each group was determined for cytospin preparations of groups A, B and C. The grain count was divided into 3 groups, the first group up to 50 grains, each cell may contain 50 grains or less, (the lightly labelled group). The second group contained cells in which each cell's grain count was up to 100 grains (the medium labelled group), while the third group contained more thans 100 grains per cell. The lightly labelled groups were pooled from two smaller class intervals [0->25 and 25->50 grains per cell], the medium labelled group was pooled from 2 smaller class intervals of [50->75 and 75->100 grains per cell], while the densely labelled group contained more than 100 grains per cell.

12. Tumour colony forming units-spleen, (Tumour.CFU-S) :

The procedure for the determination of the dose response curve for Tumour.CFU-S is as follows:

Mice were irradiated with 7.2 Gy in a Siemens-Stabiliplan X-ray machine (Siemens - Munich, West Germany)(250Kv, 14ma and 0.5mm copper filtration) in conjunction with an applicator which gave 20 cm of radiation field.

The irradiated mice were divided into groups of 20 mice each, and were kept in a warm place at 37 ^{0}C . The mice in a specified group, received a certain dose of cells per 0.25ml RPMI 1640 medium, from a freshly prepared plasmacytoma suspension. The mice were injected in the tail vein with a 1ml sterile syringe (Beckton Dickson. Ireland) and needle (25G.5/8. Beckton Dickson. Ireland). The needle was changed often (around 8-10 mice per

needle), and the benches on which the mice were injected were all cleaned with 75% ethyl alcohol.

Each mouse was transferred into a clean mouse injection box. The box is made of perspex transparent material with many holes on its sides to allow the mouse to breathe. Thus the mouse was trapped in this small box and its tail was searched for a vein, and 0.25ml of plasmacytoma suspension (without FCS) was injected carefully into the lateral cordal vein. 80 mice were used for this experiment which was performed twice. A total of 160 mice were used.

The cell dose/mouse for the different groups was as follows:

Group 1: 5×10^4 cells/0.25ml; Group 2: 7.5×10^4 cells/0.25ml; Group 3: 1×10^5 cell/0.25ml; Group 4: 1.5×10^5 cells/0.25ml

The mice were kept in separate cages, 10 mice per cage, to minimize cross-infection and were given food and water. The water contained an antibiotic, (500mg tablets-Neomycin Sulphate BP. 35×10^4 units/tablet)(Boots Plc., Nottingham, England). 2 tablets were dissolved in 2 litres of water as a final concentration for mice consumption.

Eight days after the injection of plasmacytoma cells, the mice were sacrificed under ether anaesthesia and the spleens were removed and fixed in Bouin's fixative for 24 hours. The Bouins was discarded and the spleens were kept in 75% ethyl alcohol. The spleens were examined under a stereomicroscope, and the number of colony forming unit per spleen was determined.

13. The procedure for preparing plasmacytoma cultures with UIF or Ara/C. (Effects of UIF and Ara/C on Tumour.CFU-S) :

A plasmacytoma cell suspension was prepared and divided into 4 groups 1,2,3 and 4. Group 1: contained UIF; group 2: contained UIF-Ara/C; group 3: contained Ara/C and group 4: was without UIF or Ara/C (controls).

1. Sterile bijous received 0.8ml of a plasmacytoma suspension in [RPMI 1640 without FCS] + 0.2ml UIF-STD (3ml, per bijou)

2. The same procedure as above for the UIF cultures in group 1. Groups 1 and 2 were incubated for 4 hours at 37 °C, 5% CO_2, in a Leec incubator. Following the 4 hour incubation, the cultures in group 1 and 2 were centrifuged for 7 minutes at 1.4 x 10^3 RPM in a (MSE. FISON) bench centrifuge. The supernate was discarded, and the cultures in group 1 were resuspended gently in 3ml of prewarmed 37 C, RPMI 1640, while cultures of group 2 were resuspended in 2.7ml of RPMI 1640 + 60ug of Ara/C in 300ul of RPMI 1640. Both sets of tubes were incubated for 1 hour at 37 °C in a Leec incubator. The Ara/C [Cytarabine.Cytostar. Upjohn Pharmaceuticals. Sussex, U.K.], was used at 20ug/ml of plasmacytoma suspension.

3. Sterile bijous as described above received 3ml of plasmacytoma suspension in RPMI 1640 without FCS.

4. The same procedure as above in group 3

Groups 3 and 4 were incubated for four hours at 37 °C ,5% CO_2in a Leec incubator. Following the 4 hour incubation, the cultures were centrifuged as described above for groups 1 and 2, and resuspended gently in RPMI 1640. Group 3 received 2.7ml of RPMI

1640 + 60ug of Ara/C in 300ul of RPMI 1640; while group 4 received 3ml of RPMI 1640 without Ara/C. Both sets of tubes of groups 3 and 4 were incubated for 1 hour at 37 °C, 5% CO_2 in a Leec incubator.

Following the 1 hour incubation, all sets of tubes in groups 1,2,3 and 4 were centrifuged in an (MSE.FISON) refrigerated bench centrifuge for 7 minutes at 1.4 x 10^3 RPM. The supernate was discarded and the cell pellet in each tube was resuspended gently in cool fresh RPMI 1640 medium. The cell suspension of each group was adjusted to a final concentration of 4 x 10^5 cell/ml. The final cell suspension of each group was used in 0.25ml aliquots; administered intravenously in the tails of the irradiated mice as described previously. Each plasmacytoma suspension group was tested in 20 mice, a total of 80 mice were used for this experiment, which was performed twice, (160 mice).

14. The procedures for the metaphase arrest experiment in vivo:

Vincristine Sulphate or Oncovin Sulphate was supplied in a sterile vial containing 1mg of "ONCOVIN SULPHATE", (Lilly Pharmaceuticals, Basingstoke, U.K.), supplied with a sterile vial containing 10ml of sterile NaCl solution (0.9%) and 0.9% Benzyl Alcohol as preservative.

The Oncovin vial was shaken to dissolve the Oncovin Sulphate in 5ml of NaCl solution and was stored in the fridge at 4 °C, for a maximum period of 14 days. All preparations of Oncovin Sulphate were used within the 2 weeks period. Using a 1ml sterile syringe (Beckton Dickson - IRELAND), Balb/c mice were injected intraperitoneally with Oncovin Sulphate, each mouse receiving

0.25ml of the metaphase arrest agent (0.002mg/gm. body weight), (Smith et al., 1974). An optimum dose of vincristine sulphate was used, which was the concentration which arrested all metaphases for a defined period of time for that specific tissue; the dose must also prevent complete anaphase escape, (Wright and Appleton 1980). The mice were kept for the required time period for oncovin to arrest the metaphases.

Following the administration of vincristine, the tumours were removed as described previously, at the time intervals mentioned above. Each time interval was duplicated. Thus, for example, for tumour size 5, 18 tumours were used; 2 mice per each time interval. The tumours were fixed in Bouins fixative overnight and were cut along their longitudinal axis, so that the plane of the section will be providing the maximum cross sectional area and is perpendicular to the axis, (Smith et al., 1974). Routine histological procedures were followed after the Bouins fixation, which were dehydrating through various strengths of alcohol, followed by chloroform and mounting in paraplast wax. Following the wax mounting, sections were cut from the tumour using a Leitz-Wetzlar (Leitz. U.K.), microtome set at 6um thickness. The first few sections were discarded, to ensure uniformity of thickness, (Clarke 1968). The sections were mounted on glass slides and were stained with Mayers heamalum and eosin, and eventually covered with glass cover slips with balsam.

Histological quantitation:

(A) The method devised by Chalkley (Chalkley H.W. 1943) as modified by Smith et al., (1974), was used to determine the tumour section components: tumour arrested metaphases, tumour cells,

space and necrosis in a stained tumour section. The Chalkley graticule (G52, 25 point Chalkley graticule. GRATICULES LTD.,Tonbridge, Kent, U.K.) which was fitted to the microscope eyepiece, contained 25 randomly distributed points enclosed within a circle divided into quadrants; was used to analyse a tumour section under the microscope, at (10 x eyepiece)x(40 x objective) = 400 x magnification. The area of the Chalkley at 400 x magnification was 75.507 x 10 um^2. The number of points on the Chalkley grid that coincided over tumour cells , over space and over necrosis were recorded . Thus, for example, if 16 points coincided over tumour cells, 3 points over space, and 6 points over necrotic cells or fragments, the percentage for that field viewed under (400 x magnification) will be 64% for tumour cells, 12% for space, and 24% for necrotic cells or fragments. Three sections from a tumour were examined; a section was divided into 3 zones: outer, middle, and inner as viewed under the microscope.

With the aid of the Chalkley grid at 400 x magnification, the outer zone was analysed by viewing 9 random fields. In each field viewed, the proportions of tumour cells, space and necrotic cells were recorded. The number of metaphases for that field was also recorded. The procedure was repeated for the middle and inner zones. Each zone in each tumour section, gave a mean number of metaphases and a mean number for the proportion of tumour cells, necrotic cells and space. Each zone reading was scanned for a minimum of 3000 cells and was replicated in 3 sections. The tumour sections did not contain anaphases or telophases.

(B) The diameters of several hundred randomly distributed interphase nuclei in a section, irrespective of the zone, were recorded using a microscope scaler at a magnification of, (10 x

eyepiece) x 100 objective - oil immersion) 1000 x magnification, and the mean diameter was determined. The diameters of several hundred randomly distributed metaphases in a section irrespective of the zone were recorded also, and the mean diameter was determined.

(C) Each zone was scanned for an "all tumour area", free as much as possible from necrosis and space. Using an E9A graticule fixed to the microscope eyepiece the all tumour areas were observed under a magnification of 1000 x, (= [(10 x eyepiece)x(100 x objective-oil immersion)].

The area of the E9A Graticule under the 1000 magnification was 1.111×10 um^2 . For example: an outer zone was scanned for 10 such fields (all tumour fields) and the number of cells at 1000 x magnification within the E9A graticule grid was determined for the first, second, and third sections. The three means so obtained were averaged into one mean that represented the outer zone from three sections. The same procedure was repeated for the middle and inner zones.

(D) the mean number of cells obtained for a zone, represented the mean number of cells within the surface area of the tumour section. This can be converted into a mean number of cells per unit volume, using Abercrombie's equation (Abercrombie 1946) which is:

$$P = A\ (M/L+M)$$

P = Average nuclear points per section; A = Crude count of the nuclei seen in the section; M = thickness (um) of the section; L = Average diameter (um) of the nuclei.

In this case to correct the number of tumour cells:

(1) $C' = C[t/L+t]$

where C' = Average number of cells per section

C = Observed count of nuclei in the section

t = Thickness of section = 6um

L = Average length of the diameter

The average diameters obtained for the cell nuclei and metaphases are usually 79% of their true values (Abercrombie 1946). Riches et al., (1981) have introduced a further correction to Abercrombie's equation (Abercrombie 1946), whereby the average nuclear diameters of interphase nuclei (De) and metaphase figures (Dm) can be converted into true average diameters, using the following equations:

(1) $Dt = -(T-De/1.58)+1/1.58[T +1.16TDe+De]^{1/2}$ Dt= true average interphase nuclear diameter, De= average measured interphase nuclear diameter, T =section thickness

(2) $Dp = -(T-Dm/1.58)+1/1.58 [T +1.16T Dm+Dm]^{1/2}$ Dp= true average metaphase diameter, Dm= average measured metaphase diameter, T = section thickness.

The average measured interphase nuclear diameter was (6.61um+0.075); and the average measured metaphase diameter was (4.847um+0.055). Using the correction introduced by Riches et al., (1981), as shown earlier: the true average value for the nuclear diameter = 7.475um and the true average value for the metaphase diameter = 5.38um.

Applying Abercrombie's correction (Abercrombie 1946), the number of cells per unit volume and the number of metaphases per unit volume of section could be calculated, and therefore an estimate of the number of metaphases per 1000 cells.

(E) Having obtained a mean value for the number of tumour cells in an "all tumour area" for the outer zone, as has been described previously; an example, can be described for the calculation: a mean of 13.6 cells was scored under a magnification of 1000 x (oil immersion) within the E9A graticule. When using a Chalkley under a magnification of 400 x, the area that will be scanned by the Chalkley (G52; 25 point graticule) equals 75.5071 x 10 um^2, thus more cells will be scanned at the 400 x magnification when compared to that at a magnification of 1000 x. Thus the mean Expected number (Ex G52) of cells within the Chalkley G52 graticule at 400 x magnification equals the number of cells within the Chalkley G52 graticule at 1000 x magnification multiplied by a change of magnification (MF).

(1) Mean Expected number of cells per G52 or (Ex-G52) = Mean observed number of cells per E9A, 13.6 x MF-67.957 = 924.21

(2) 3 outer zones per section were scored (OZ1, OZ2, OZ3) with 9 random fields per zone scanned using a Chalkley GG52 graticule. The zone has a mean value: for the metaphases; for the proportion of tumour cells, and the remaining proportion of necrosis and space.

The mean actual number of cells per G52 Ac-G52 = (Ex-G52)x(mean % tumour cells in OZ1.

The mean % value of tumour cells in OZ1 was 77.3%,

and the mean value of metaphases in OZ1 = 11.66.

Ac-G52 = (Ex-G52)x(% tumour cells in OZ1) = 924.21 x 0.773 = 714.41 cells.

Using Abercrombie's correction (Abercrombie 1946) and Riches' correction (Riches et al., 1981), the number of cells per unit volume can be calculated for OZ1,

P = A [M/L+M], or

C' = C [t/L+t] = Ac-G52 = G52 [t/Dt+t] = 714.41[6/7.475+6]

= (714.41)x(0.445) = 318.1 tumour cells.

The number of metaphases per unit volume can be calculated also:

M' = M [t/L+t] = M [t/Dp+t] = 11.66[6/5.38+6] =

(11.66)x(0.527) = 6.11 metaphases.

Thus a value in terms of unit volume of tumour of 6.11 metaphases per 318.1 tumour cells has been determined for OZ1. The corrected number of metaphases per 1000 tumour cells = 6.11 x (1000/318.1) = 19.2. The same procedure was applied for other zones. Having obtained the corrected number of metaphases per 1000 tumour cells for OZ1, OZ2 and OZ3, a pooled mean for these 3 sets of values can be determined.

In a similar way the corrected number of metaphases per 1000 cells can be determined: using the mean actual value of cells scored in a zone, the mean number of metaphases determined for a zone can be corrected to the number of arrested metaphases per 1000 tumour cells.

The ratio of the corrected metaphase counts to the corrected number of tumour cells per zone is expressed as follows:

$M'/C' = M[t/Dp+t]/C[t/Dt+t]$

$= M/C[t/Dp+t \times Dt+t/t]$

$= M/C[13.475/11.38]$

$= M/C[1.18]$

[M'/C'](1000) = [mean number of metaphases in a zone/Ac-G52](1000)(1.18)

= [11.66/714.41](1000 x 1.18) = 19.25 metaphases per 1000 tumour cells. Each pooled corrected metaphase per 1000 cells is corrected to (1+Imet) and to $\log_{10}$(1+Imet). Metaphase accumulation in the tumour was plotted as the "Collections Function" at half hour intervals, including zero time, to a maximum time of 4 hours. The y-axis on the graph represents $\log_{10}$ (1+Imet), the x-axis represents the time period. The formula for the "Collection Function" was developed by Puck and Steffen, (1963).

$\log_{10}(1+N(m)] = 0.301/T\ (tm+t)$

Similarly, $\log_{10}$ (1+Imet) = 0.301/Tca (tm+t), when the growth fraction is unknown.

KB = 0.301/Tca and Tca = 0.301/KB

The delay period observed in the action of a stathmokinetic agent to arrest metaphases in some tissues, (Puck and Steffen 1963, Smith et al., 1974, Nome 1975, Wright and Appleton 1980), must be taken into account when measuring the birth rate from the slope of the

line. The data of the metaphase "Collection Function" were plotted against time after vincristine injection and were analysed using a computer, (VAX Computer, University of St. Andrews) using a weighted and unweighted least squares technique, (Wright and Appleton 1980). The birth rate (KB) is the regression coefficient produced by the least squares method, from which the apparent cell cycle time (Tca) was estimated according to the "Collection Function" of Puck and Steffen (1963).

The mean birth rate is expressed as: cells per cell per hour. The cell production rate per 1000 cells (Smith et al., 1974) can be estimated by multiplying the birth rate (KB) by 1000, and will be expressed as: cells per 1000 cells per hour. The metaphase duration can also estimated from the "Collection Function" of Puck and Steffen, (1963):

t met = intercept of regression line on metaphase collection axis / KB

The cell loss factor (ϕ), (Steel 1968) was estimated from the Tca and (Td), tumour doubling time:

as $\phi = 1-Tca/Td$.

15. Measurement of tumour growth rates:

The animals used were Balb/c mice of both sexes and some were "nude" mice with a Balb/c background. The mice used for the experiments were between 7 - 10 weeks of age. The mice were housed in plastic cages with saw dust, and were given water and mice food diet. The "nude" mice were housed in a separate room, and their

drinking water contained (Neomycin sulphate B.P. Boots PLC U.K.); 2 tablets (500 mg each) which were dissolved in 2 litres of water. When handling the "nude" mice, a face mask was worn together with a clean laboratory coat for use in the "nude" mice room only. The mice were in a air-conditioned environment.

A cell suspension of plasmacytoma free of red blood cell was prepared in RPMI and was adjusted to a concentration of 5×10^6 cells/ml. Using 1ml sterile syringe (Beckton Dickson) fitted to a sterile needle (23-1 1/4, Beckton Dickson. Ireland) each mouse was injected subcutaneously in the inguinal region (which was swabbed in 70% ethyl alcohol), with 0.1ml of the cell suspension (5×10^5 cells) per mouse. Different cell doses were also injected in Balb/c mice, when required for some experiments. The mice were left in cages with food and water for a period of two weeks when the tumours in most instances reached a maximum size, after which the mice were killed. The mice were observed daily for tumour growth (nodule formation) in the inguinal region. When a tumour reached size group 3 (100 mm^3; or tumour group size 3: equal to ball bearing size 3.5-3.8) which were fairly palpable, tumour size was recorded (and taken as V=0 at T=0, intial volume=V0), and thereafter tumour growth was followed regularly and recorded every 48 hours (V1, 2, 3 at T1, 2, 3...etc....) until reaching (Vmax) a maximum volume desired for the experiment, or otherwise until reaching a maximum volume tolerated by the mice and this was tumour size 9 after which the mice were killed. The tumour volume was assessed in terms of steel ball bearings of fixed diameters. The tumour and the ball bearing were felt by the same hand (thumb and forefingers) and tumour volume was recorded accordingly. The ball bearings were obtained from (Hoffman Manufac. Plc. Essex, U.K.) and were covered with chamois leather to simulate mouse skin.

The steel ball bearings were of the following diameters: 3/16 up to 12/12 inch. The ball bearing size was graded as follows: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, with the corresponding ball bearing volume (.mm^3) of: 56.55, 134.11, 260.55, 452.28, 717.75, 1071.71, 1527.27, 2095.57, 2789.07, 3619.9. The tumour volume was calculated as follows:

$\pi 4/3\ r^3 = \pi d^3/6$

(1/16)in = 1.5875mm

Tumour size 3, therefore, will have the following volume: $[3(1.5875)]^3 x \pi/6 = 56.55\ mm^3$.

The tumours were measured as accurately as possible and were graded as follows:

tumour size = 3, 3+, 3.5, 3.5+, 4, 4+, 4.5, 4.5+, 5, 5+, 5.5, 5.5+, 6, 6+, 6.5, 6.5+, 7, 7+, 7.5, 7.5+, 8, 8+, 8.5.

The corresponding tumour volume (.mm^3) was as follows: 56.55, 68.66, 89.83, 114.87, 134.11, 155.25, 190.08, 231.7, 260.55, 293.32, 347.4, 408.85, 452.28, 498.16, 575.18, 658.75, 717.75, 781.66, 883.26, 994.36, 1071.71, 1155.2, 1286.38.

The growth rate of tumours was estimated by using exponential regression analysis preprogrammed in a Casio-fx3600P electronic calculator.

The formula for exponential regression was:

$Vt = Vo \exp mt$

$\ln Vt = mt + \ln Vo$

The volume doubles in a period = Td, therefore the volume at t = Td is, Vt = 2V0; therefore: $2 = \exp mTd$ i.e. $2V0 = 0 \exp mTd$

$\ln 2 = mTd$

$Td = m/\ln 2$

m = slope of the curve, which is the growth rate (KG)

V0 = initial tumour volume

Vt = volume at time t

Td = tumour doubling time

lnV0 = intercept

t = time of measurement.

16. Statistical Procedures:

The statistical calculations were based on the methods described by von Fraunhofer and Murray (1976), Kirk (1969), Parker (1979), Snedecor and Cochran (1967)and Sokal and Rohlf (1981).

The t test comparison of two sample means :

t = difference of means/standard error of difference between means.

Analysis of variance (F.ratio) = treatments/error; (treatments = mean square between groups), (error = mean square within groups).

Dunnett's test for comparisons involving a control mean (Kirk 1969): Comparing a number of treatment levels with a control condition. The difference (d') that a comparison must exceed in order to be declared significant is given by:

$$d' = tDa/2;k,v\left(2(MS\ error)/n\right)^{1/2}$$

where tDa/2;k,v is the two-tailed value obtained from Dunnett's table.

a = the error rate experiment wise

2 = two tailed test

k = number of treatments, including control

v = degrees of freedom associated with MS error.

Multiple range test, using Shortest Significant Ranges (S.S.R.) (Parker 1979): The means are arranged in order of magnitude and a series of S.S.R's is calculated for testing differences between pairs of means according to their relationship in the size order.

1. Analysis of variance = treatments/error

2. S.E. of treatment mean = $\sqrt{\text{error/d. freedom}}$

3. Q is found in the Studentized Range table

4. The values of Q were determined for the number or degrees of freedom for error and for k, (K-1)... etc. treatments.

5. The S.S.R's for k, (k-1)... groups were computed as Q [error/d. freedom]

6. Having arranged the treatment means in order of magnitude, the difference between the largest and the smallest means was tested against the S.S.R. for k treatments. If the difference was not significant the testing must be stopped, but if it was significant, the testing of the difference between the largest and the next to the smallest must be determined as described by Parker (1979).

Regression Analysis:

$Y = a + bX$

a = intercept

b = regression coefficient

The slope or regression coefficient for tumour growth: m or $KG = \sum t(\ln Vt)/\sum t^2$

Residual variance: $SR = 1/N-2[\ (\ln Vt)^2 - (\sum(t \ln Vt)^2 / \sum t\ ]$

$SR = \sqrt{SR^2}$, S.E. of $m = SR/\sum t^2$

The t test for growth rate comparisons:

$$m1-m2/\sqrt{(S.E.m1)^2 + (S.E.m2)^2}$$

The S.E. of Td = (S.E. of m/m^2)ln2

The t test comparison for Td:

$$Td1-Td2/\sqrt{(S.E.Td1)^2 + (S.E.Td2)^2}$$

The S.E. of Tca = S.E. of $KB(0.301)/KB^2$

The t test comparison for Tca:

$$Tca1-Tca2/\sqrt{(S.E.Tca1)^2 + (S.E.Tca2)^2}$$

The standard error of L.P.(latency period) = S.E. of m = $(lny-lnc)/m^2$. c = intercept

The latency period was estimated from the regression curve as L.P. = lnVt-lnc/m

The S.E. of tm = $\sqrt{(\ c^2 \times var.Tca+(Tca)^2 \times var.c\ )}$

c = intercept

The t test for tm comparisons:

$$tm1-tm2/\sqrt{(S.E.tm1)^2 + (S.E.tm2)^2}$$

The S.E. of ϕ cell loss factor = S.E.Tca/Td

$$= (Tca)^2/(Td)^4[var.Td] + 1/(Td)^2[var.Tca]$$

The T test comparisons of ϕ cell loss factor = $\phi 1-\phi 2/\sqrt{(S.E.\phi 1)^2+(S.E.\phi 2)^2}$

CHAPTER THREE

RESULTS

3.1. IN VITRO CULTURES: ASSESSMENT OF THE EFFECTS OF FCS AND OTHER SERA ON CELL PROLIFERATION.

3.1.1. The relationship between the cell number per microtitre well and the uptake of 125IUDR by cells in short term cultures of 1 and 4 hours.

Experimental procedures:

A cell suspension of plasmacytoma cells was prepared as described previously in materials and methods. Microtitre wells received the following cell concentrations per well: 1×10^4, 5×10^4, 1×10^5 and 5×10^5; each microtitre well, received 0.2 uci of 125IUDR. The final volume per well was 200 ul; each cell dose was replicated 5 times. Culture preparations were incubated at 37 °C, 5% CO_2 for 1 hour, and 4 hours . Some cultures were not supplemented with FCS, while other cultures were supplemented with 10% of either heat inactivated FCS (FCS-HI), or normal FCS (Active FCS). Similarly, a cell suspension of mouse thymocytes was prepared , and was dispensed into microtitre wells at the following cell concentrations per well: 1×10^4, 2.5×10^4, 5×10^4, 1×10^5, 1.5×10^5, and 2.5×10^5. The cell cultures were supplemented with 10% FCS-HI; each culture received 0.2 uci 125IUDR. Cultures were incubated at 37 °C, 5% CO_2, for 4 hours as described previously. Following the incubation, the uptake of isotope by cell cultures

was assessed as described previously. The percent inhibition of isotope uptake by cell cultures was calculated as follows: [A - B] / [A]

A = mean cpm of control cultures .

B = mean cpm of test cultures .

Results:

The uptake of 125IUDR by cell cultures, expressed as mean counts per minute (mean cpm), has increased as the cell dose/culture increased, (Figures/Tables 1.1.1, 1.1.2, 1.1.3). Curve [A], (Figures 1.1.1 and 1.1.2) represents cultures, cultured without FCS; curve [B] represents cultures, cultured with FCS-HI; while curve [C] represents cultures, cultured with active FCS. The uptake of 125IUDR by cell cultures has also increased as the period of incubation has increased to 4 hours, (Figure 1.1.2 compared to Figure 1.1.1), at all cell doses. The results (Figure/Table 1.1.2) have shown that the highest uptake of 125IUDR was in the 1×10^5 cell dose group, cultured without FCS; which was significantly different from its counterparts which were cultured with FCS-HI, ($0.02>P>0.01$), or with Active FCS, ($P<0.01$). The difference in isotope uptake with and without FCS-HI was approximatly 32% and the difference in uptake with and without Active FCS was 37%. Similarly, in the 5×10^4 cell dose group, the highest uptake of 125IUDR was by cells cultured without FCS; which was significantly different from the isotope uptake by cells cultured with FCS-HI,($P<0.01$); and from the uptake by cells cultured with Active FCS, ($P<0.01$). The difference in isotope uptake with and without FCS-HI was approximatly 46% and the difference in isotope uptake with and without Active FCS was 51%.

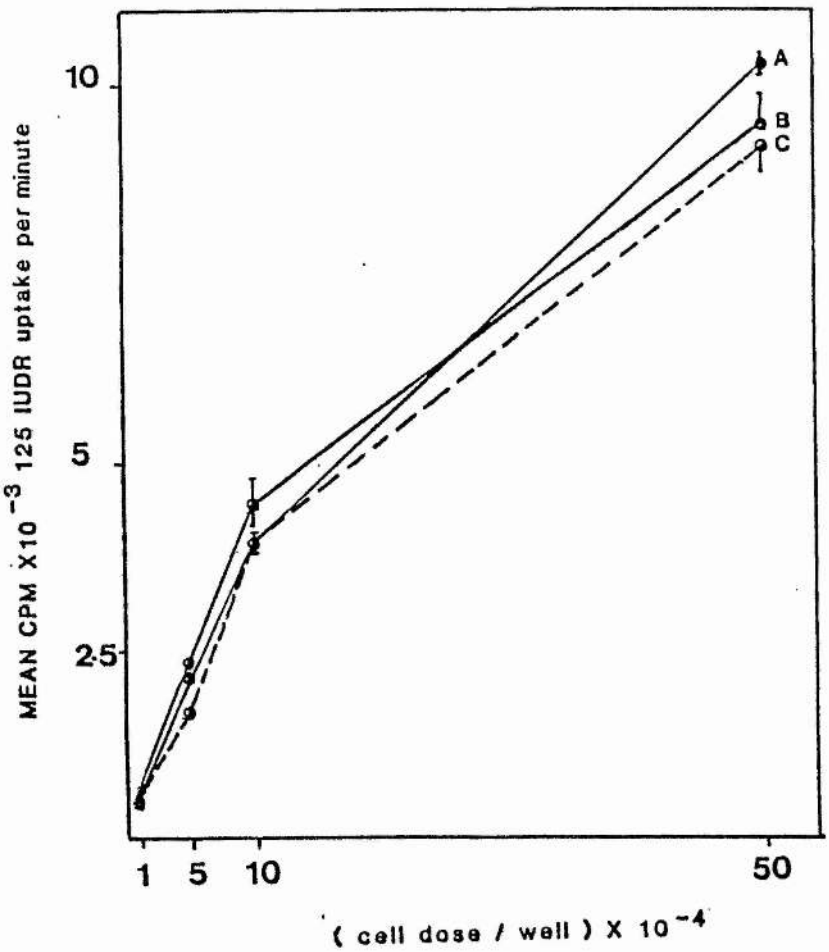


FIGURE 1.1.1: The relationship between the cell dose per culture well and the uptake of 125IUDR by cell cultures, cultured for a 1 hour incubation period.
Plasmacytoma cultures, cultured with; A: without FCS, B: with FCS -HI, C: with active FCS .

Table 1.1.1:

The relationship between cell number per well and the uptake (expressed as mean counts per minute) of 125IUDR by plasmacytoma cultures for a 1 hour incubation period.

a: cells cultured in medium without FCS.
b: cells cultured in medium and heat inactivated FCS.
c: cells cultured in medium and active FCS.

Cell dose/well	1×10^4	5×10^4	1×10^5	5×10^5
Mean cpm ± S.E.	a: 496 ± 83 b: 561 ± 19 c: 554 ± 19	2112 ± 17 2309 ± 18 2369 ± 51	3955 ± 44 4472 ± 215 4527 ± 271	10311 ± 92 9487 ± 329 9231 ± 364

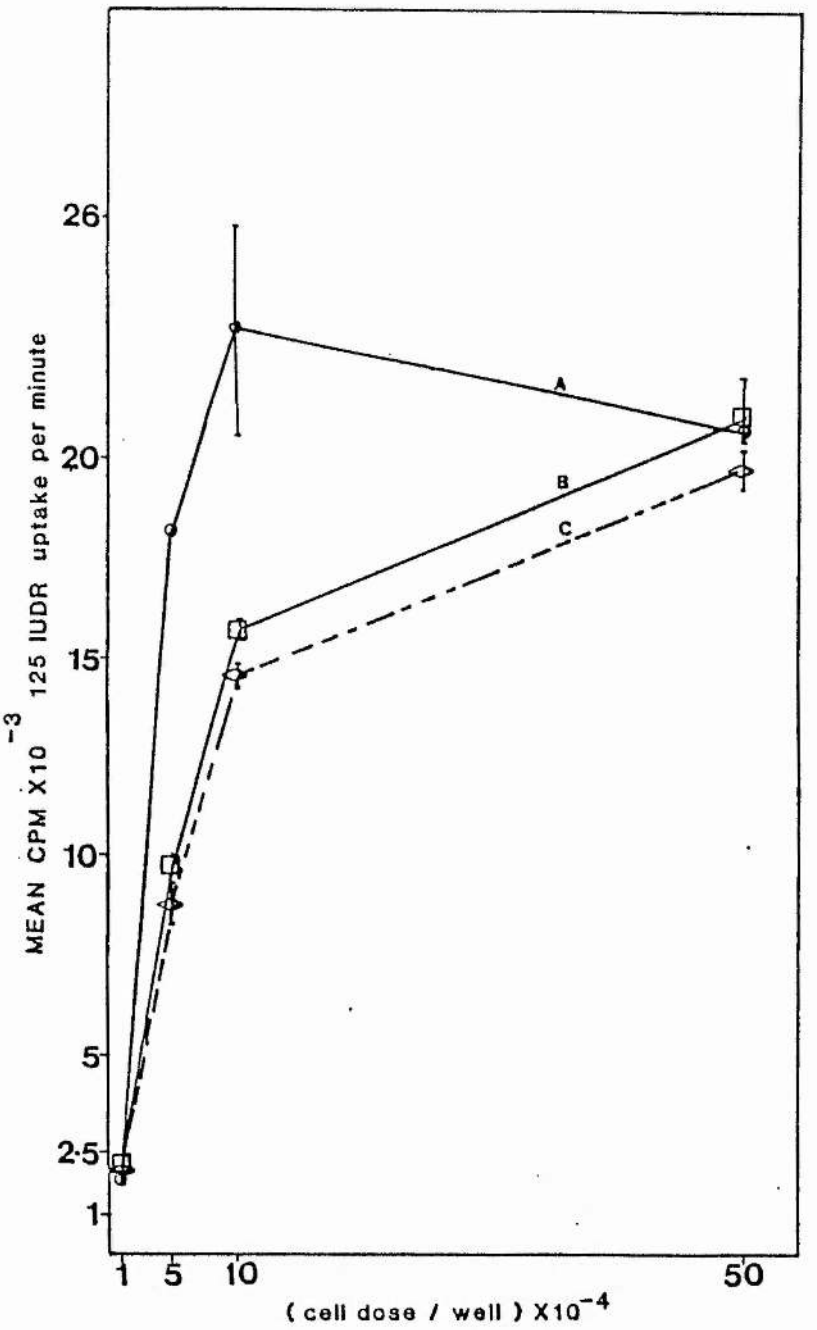


FIGURE 1.1.2; The relationship between the cell dose per culture well and the uptake of 125IUDR by cell cultures, cultured for a 4 hours incubation period.
Plasmacytoma cultures, cultured with; A: without FCS, B: with FCS -HI, C: with active FCS .

Table 1.1.2:

The relationship between cell number per well and the uptake (expressed as mean counts per minute) of 125IUDR by plasmacytoma cultures for a 4 hour incubation period.

a: cells cultured in medium without FCS.
b: cells cultured in medium and heat inactivated FCS.
c: cells cultured in medium and active FCS.

Cell dose/well	1×10^4	5×10^4	1×10^5	5×10^5
Mean cpm ± (S.E.)	a: 1774 ± 70 b: 2269 ± 20 c: 2061 ± 111	18148 ± 83 9745 ± 184 8831 ± 404	23278 ± 2453 15765 ± 154 14624 ± 274	20680 ± 293 21072 ± 1037 19754 ± 518

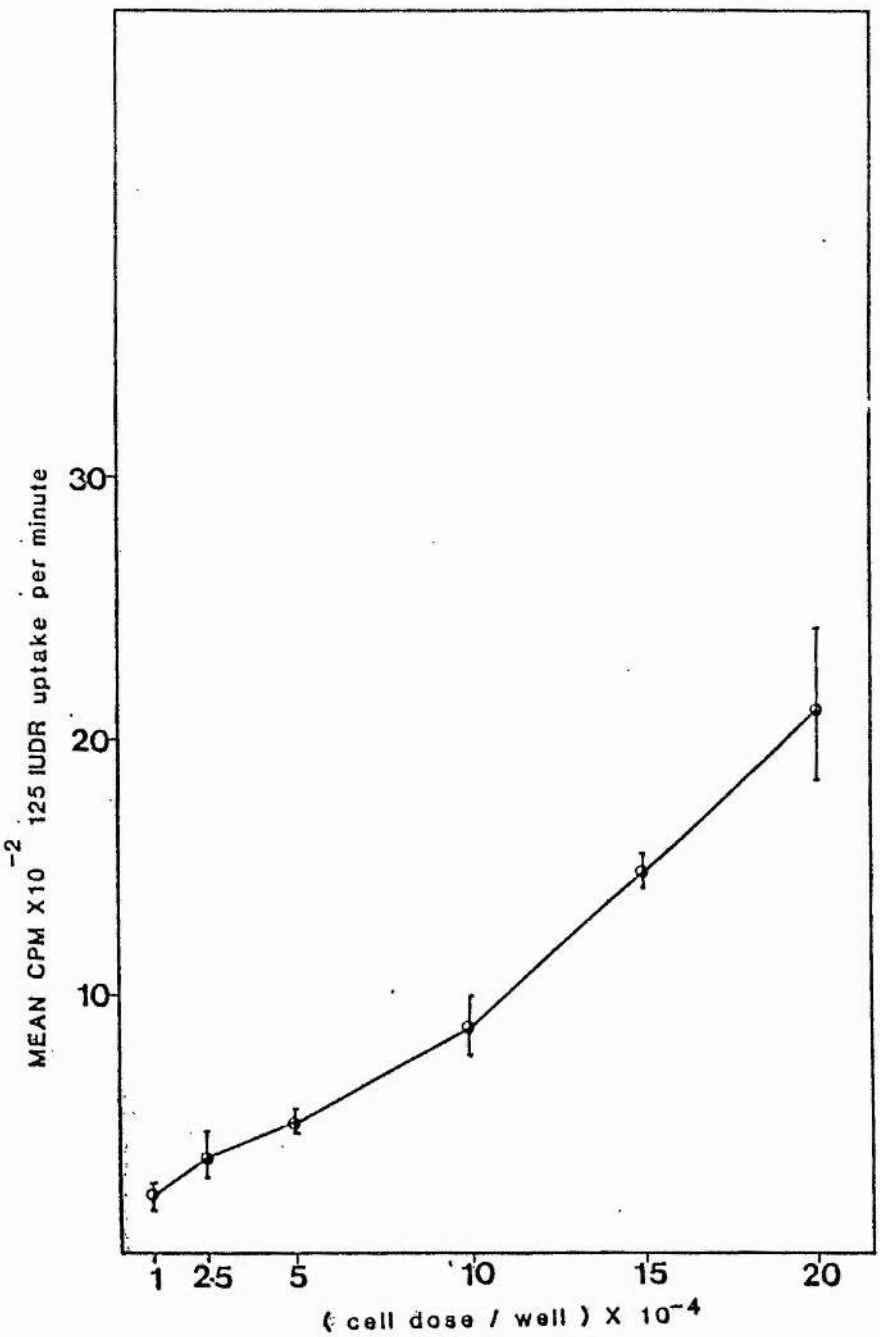


FIGURE 1.1.3: The relationship between the cell dose per culture well and the uptake of 125IUDR by mouse thymocyte cultures, cultured with FCS-HI for 4 hours .

Table 1.1.3: The relationship between thymocyte dose per well and the uptake of 125IUDR for a 4 hour incubation period. The values are expressed as mean counts per minute with ± S.E.

Cell dose/well	mean cpm
1×10^4	228 ± 31
2.5×10^4	392 ± 78
5×10^4	508 ± 33
1×10^5	878 ± 115
1.5×10^5	1489 ± 64
2×10^5	2132 ± 286

In the 5 x 10^5 cell the uptake of isotope by cultures, cultured without FCS or with FCS-HI, or Active FCS, was not significantly different (P>0.05., respectively). The highest uptake of isotope (Table 1.1.2) was by cell dose group 1 x 10^5, followed by cell dose group 5 x 10^5 both of which were cultured without FCS. However, there was not any significant difference (P>0.01), between both culture groups.

As the mean cpm of the 1 x 10^5 cell dose was on the linear portion of the curve, it was the dose chosen for in vitro short term cultures. The highest mean cpm for the thymocytes, (Figure 1.1.3) was at a concentration of 2 x 10^5 cells/well, but since the standard dose for the plasmacytoma was chosen at the 1 x 10^5 cells/well; the dose for the thymocytes for short term cultures was also set at 1 x 10^5 cells/well to standardise the cell culture procedures.

The uptake of isotope by the respective cell cultures at different cell doses per culture, cultured with or without FCS was assessed. The uptake of isotope was verified in terms of the optimum cell dose per culture. For plasmacytoma the uptake of 125IUDR was assessed at two incubation periods (1 and 4 hours), in three different culture environments, (without FCS, Active FCS and FCS-HI 56 ℃). The uptake of 125IUDR by plasmacytoma cultures, varied with the culture environment, the incubation period and cell dose per culture. The highest uptake of isotope, therefore, was at the 1 x 10^5 cell dose, cultured for 4 hours without FCS.

3.1.2: The relationship between the concentration of FCS per culture and the uptake of 125IUDR by cell cultures.

Experimental procedures:

Cell suspensions of plasmacytoma, mammary adenocarcinoma and mouse thymocytes were prepared, and dispensed into microtitre wells at a concentration of 1 x 10^5 cells/well as described previously. Cells were cultured without FCS, and were also cultured with the following concentrations of FCS-HI : 5%, 10%, 15% and 25% ; each culture received 0.2 uci of 125IUDR and was replicated 5 times. The final volume per well was 200 ul. Plasmacytoma cultures were incubated as described previously; for the following periods of time : 0.5, 1, 2, 4, 6, and 8 hours; mammary adenocarcinoma for 2, 4, 6 and 8 hours, while mouse thymocyte cultures were incubated for 1, 2, 4 and 6 hours. In another experiment, plasmacytoma cultures were cultured for a 4 hour period with a similar set of FCS concentrations. The FCS was of two types, heat inactivated at two temperatures: 56 °C, 0.5 hour and 66 °C, 12 minutes.

The uptake of isotope by cultures was assessed as described previously.

Results:

Each mean cpm in the 56 °C group was compared to its counterpart in the 66 °C group. A t- test comparison, between the mean cpm of cultures, cultured with FCS-56 °C and their counterparts cultured with FCS-66 °C, was not significant; although the mean cpm of cultures, cultured in 5% and 10% FCS-66 °C were relatively higher than the FCS-56 °C group, (Table 1.2.1).

Table 1.2.1: Mean uptake (cpm) of 125IUDR by plasmacytoma cells in FCS heated at 56C° and 66C°. 4 hour incubation period. ± Standard error values are included.

	Mean CPM ± S.E.				
FCS Concentration	(0%)	5%	10%	15%	25%
FCS 56C°	8199 ± 320	6038 ± 599	5865 ± 258	5346 ± 288	4144 ± 128
FCS 66C°	8199 ± 320	7722 ± 204	6165 ± 441	5339 ± 294	3742 ± 91

The uptake of 125IUDR by plasmacytoma cultures was reduced as the FCS concentration per culture has increased stepwise from 5% to 25%. This was true for all time periods of incubation, (Table 1.2.2.1A and Figure 1.2.2.1). The percent inhibition of 125IUDR uptake with increasing serum concentration has also been described, (Table 1.2.2.1B). The inhibition of 125IUDR uptake by cell cultures, was a function of the FCS concentration, and has increased as the FCS concentration increased; for all incubation periods. For each serum concentration there was a relative decrease in inhibition with increasing period of incubation, from 2 hours to 8 hours, except for some fluctuations in the values between 0.5 and 1 hour. An analysis of variance (Anovar.), and Dunnett's test for comparisons of means involving a control mean, were performed to assess the significance of isotope uptake inhibition, by each serum concentration on its respective culture, for each incubation period (Table 1.2.2.1A), were as follows :

Analysis of variance:

Anovar; F.ratio and Dunnett's test (2 tail, P= .01) between the mean cpm of the following culture groups : controls (0% FCS), 5%, 10%, 15% and 25% FCS. The mean cpm of cells which were cultured without FCS, was significantly different from the mean cpm of cultures, cultured with 5%, 10%, 15% and 25% FCS.

0.5 hour: F.ratio = 26.13, (P<.01); 1 hour: F.ratio = 46.09, (P<.01); 2 hour: F.ratio = 76.70, (P<.01) 4 hour: F.ratio = 240.36, (P<.01); 6 hour: F.ratio = 34.94, (P<.01); 8 hour: F.ratio = 25.11, (P<.01).

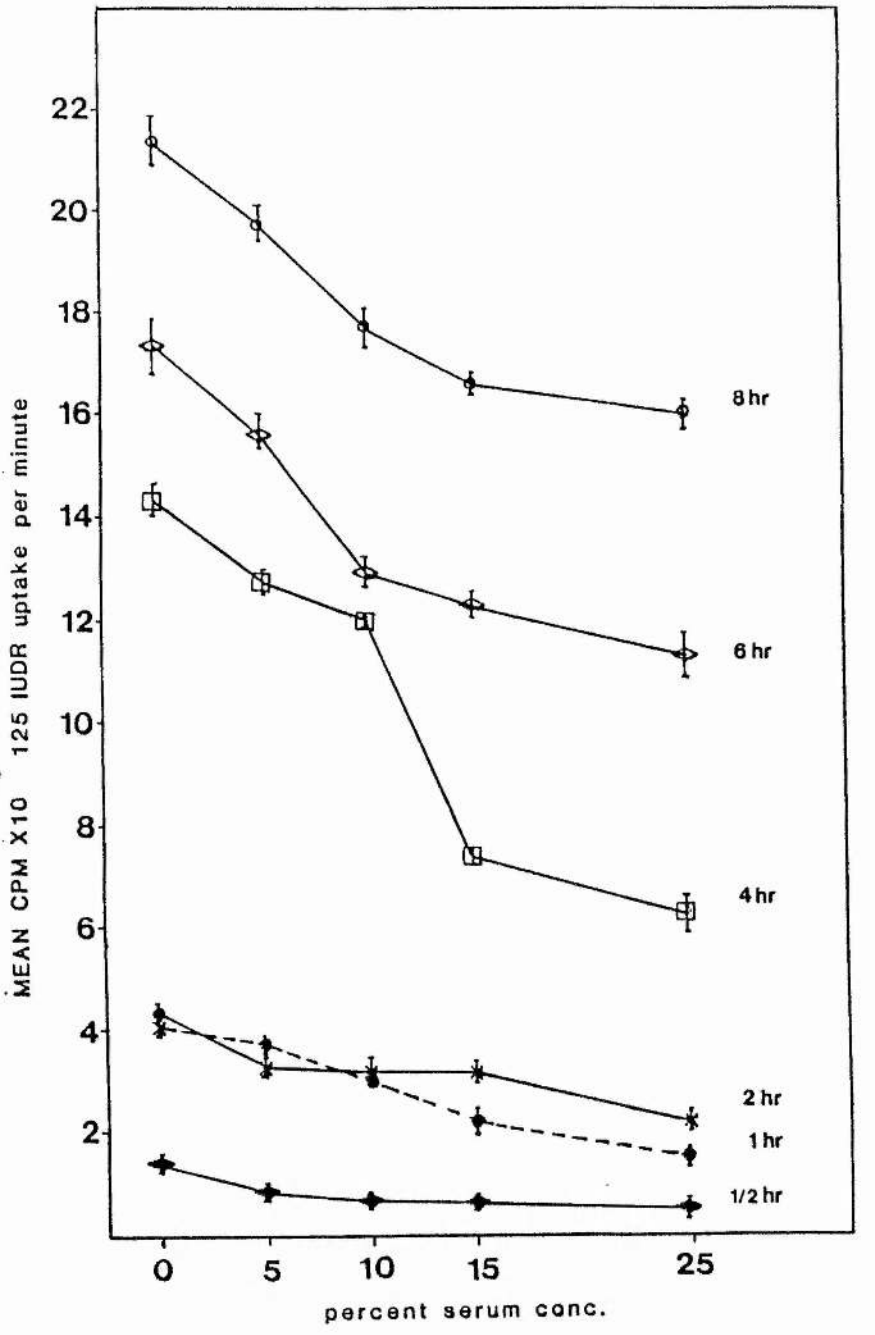


FIGURE 1.2.2.1: The uptake of 125IUDR by plasmacytoma cultures cultured with different concentrations of FCS-HI, for different periods of incubation time .

Table 1221A: The uptake of 125IUDR(expressed as counts per minute) by plasmacytoma cultures, cultured in several concentrations of FCS, for different incubation periods. The ± S.E. values are included.

FSC serum concentration %	M (0 %)	5%	10%	15%	25%
	Mean cpm and ± S.E.				
Time (hrs) = 0.5	1442 ± 42	922 ± 54	783 ± 84	725 ± 42	629 ± 76
1	4147 ± 93	3706 ± 144	3080 ± 116	2226 ± 280	1527 ± 47
2	6883 ± 332	4310 ± 208	3331 ± 82	3193 ± 195	2233 ± 81
4	14424 ± 324	12835 ± 203	11249 ± 91	7460 ± 283	6339 ± 117
6	17474 ± 545	15628 ± 460	12975 ± 335	12354 ± 326	11387 ± 418
8	21411 ± 529	19792 ± 431	17740 ± 434	16595 ± 482	16089 ± 326

m = control cultures without FCS.

Table 12 21B: The percentage of 125IUDR uptake inhibition by the following (FSC.HI) concentrations: 5%, 10%, 15% and 25% as shown in Table 1.3.1A for the duration of their respective incubation period in hours 0.5, 1, 2, 4, 6, 8. The ± S.E. is included.

Percent serum concentration	Incubation time (hours) 0.5	1	2	4	6	8
			Percent Inhibition ± S.E.			
5	36 ± 4.1	10.63 ± 3.5	37.38 ± 3	11.0 ± 1.4	10.50 ± 2.64	7.5 ± 2.01
10	45 ± 5.8	25.70 ± 2.8	51.50 ± 1	22.0 ± 0.6	18.17 ± 4.2	17.1 ± 2.03
15	49 ± 2.8	46.30 ± 6.7	53.60 ± 2.8	48.2 ± 1.96	29.20 ± 1.8	22.4 ± 2.25
25	56 ± 5.2	63.10 ± 1	67.50 ± 1	56.0 ± 2.4	34.80 ± 2.4	24.8 ± 1.52

The cell viability counts (3 replicates each) were assessed for cells cultured without FCS (0% FCS = culture controls), and with FCS at 5%, 10%, 15% and 25% per culture for 2, 4 and 8 hours incubation periods. The cell viabilities were not significantly different from each other; (t-test, $P > .01$).

The inhibition of 125IUDR uptake by mamammary adenocarcinoma cultures has increased as the FCS concentration per culuture increased, for each incubation period, (Figure/Table 1.2.2.2). The inhibition of isotope uptake has increased for each serum concentration, as the period of culture incubation increased. However, at 2 hours of incubation, 5% and 10% FCS stimulated isotope uptake by respective cultures, the uptake of isotope by cultures, cultured with 5% FCS was significantly different from the uptake of isotope by control cultures (0% FCS), ($P < .01$); while the uptake of isotope by cultures, cultured with 10% FCS, was not significantly different ($P > .05$), from the uptake of isotope by control cultures. The change in isotope uptake by cultures, cultured with 15% and 25% FCS, was not different ($P > .05$), from the uptake of isotope by control cultures. At 4 hours of incubation, the effects of 5% to 15% serum stimulated the uptake of isotope by respective cultures , while the effects of 25% FCS on isotope uptake by respective cultures, were inhibitory. The uptake of isotope by cultures, cultured with 5% serum was different from the uptake of isotope by control cultures ($0.05 > P > 0.01$); while the difference in isotope uptake by cultures, cultured in the other three serum concentrations was not different from the uptake of isotope by control cultures ($P > .05$). At 6 hours of incubation the uptake of isotope by cultures, cultured with 5% serum was stimulated ($0.05 > P > .01$), while the uptake of isotope by

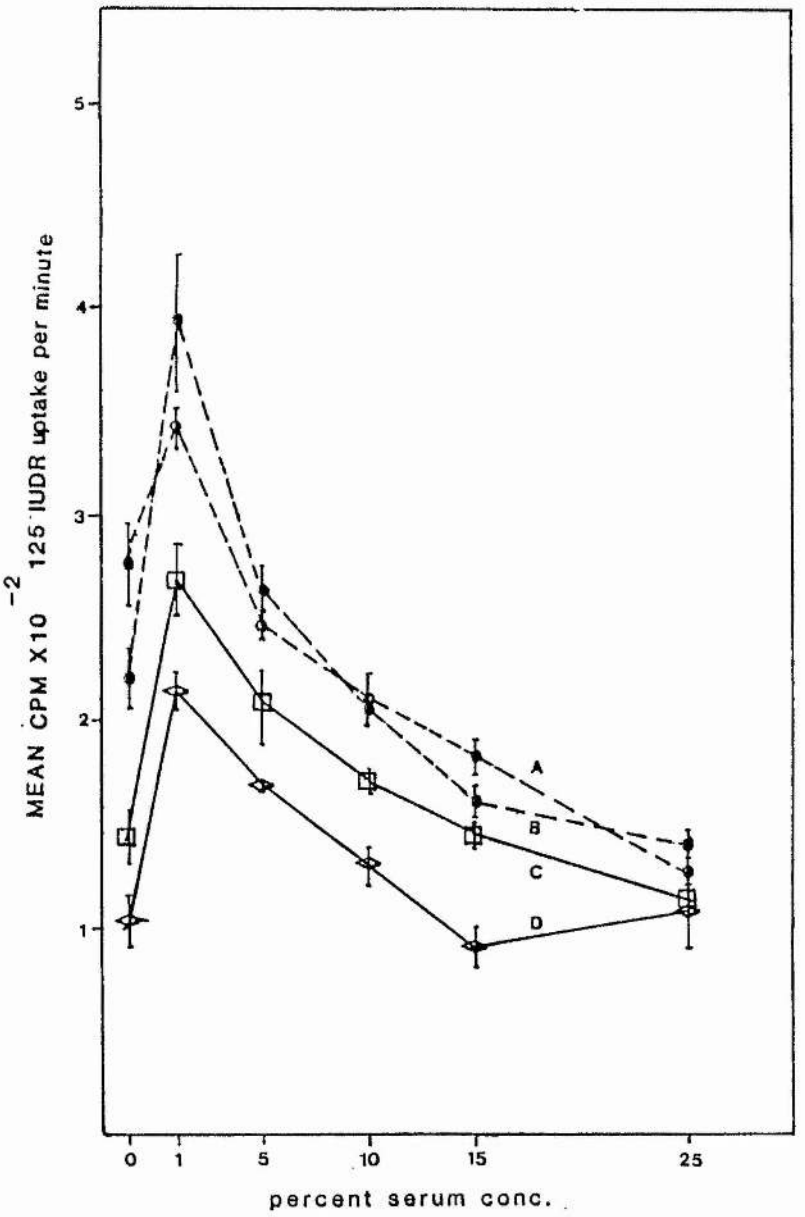


FIGURE 1.2.2.2: The uptake of 125IUDR by mammary adenocarcinoma cultures, cultured with different concentrations of FCS-HI for different periods of incubation time.
A: 8 hours, B: 6 hours, C: 4 hours, D: 2 hours .

Table 1.2.2.2: The mean uptake of 125IUDR by mammary adenocarcinoma cells, expressed as counts per minute (cpm) and as percent inhibitions, cultured in different concentrations of FCS, for different incubation periods. Standard error values are included.

Percent Serum Concentration	Incubation time (hours) and mean cpm (± S.E.)			
	(2)	(4)	(6)	(8)
0	101 ± 11	143 ± 13	221 ± 14	275 ± 20
5	169 ± 1	209 ± 17	261 ± 8	247 ± 5
10	131 ± 7	170 ± 5	206 ± 7	206 ± 15
15	91 ± 9	147 ± 5	160 ± 7	182 ± 8
25	110 ± 22	112 ± 6	140 ± 14	127 ± 14
Percent Serum Concentration	Incubation time (hours) and percent inhibition ± S.E.			
	(2)	(4)	(6)	(8)
0	-	-	-	-
5	+ 66.20 ± 1.72	+ 46.13 ± 12.20	+ 18.16 ± 3.82	- 10.26 ± 2.14
10	+ 28.94 ± 7.73	+ 19.16 ± 3.55	- 6.92 ± 3.38	- 25.13 ± 5.63
15	- 9.75 ± 9.03	+ 2.79 ± 3.82	- 27.27 ± 3.46	- 33.77 ± 3.12
25	+ 8.26 ±22.13	- 21.13 ± 4.55	- 36.43 ± 3	- 53.59 ± 5.15

+ = stimulation of uptake
- = inhibition of uptake
} 125IUDR

cultures, cultured with 10%, 15%, and 25% FCS, was inhibited; the uptake of isotope by cultures, cultured with 10% serum was not significantly different from the uptake of isotope by control cultures, ($P > .05$); while the uptake of isotope by cultures, cultured with 15 and 25 percent FCS was different ($P < .01$), from the uptake by control cultures.

At 8 hours of incubation, the effects of the respective serum concentrations on the uptake of isotope by cultures, were inhibitory. The uptake of isotope by cultures, cultured with 5% FCS was not significantly different from the uptake by control cultures ($P > .05$). The uptake of isotope by cultures, cultured with 10% FCS was different ($0.05 > P > .01$), so was the uptake by cultures, cultured with 15% and 25% FCS ($P < .01$). The cell viability at 8 hour in 25% serum was not significantly different ($P > .05$) from cell viability of cultures, cultured without FCS.

The inhibition of isotope uptake by mouse thymocyte cultures has increased as the serum concentration per culture increased, in each incubation period, (Figure/Table 1.2.2.3). The uptake of isotope by cultures, cultured with 5% and 10% FCS has increased with increasing incubation periods (1-4 hours) with a slight decrease at 6 hours. The change in isotope uptake by cultures, cultured with 5% and 10% FCS, for all incubation periods was significantly different from the uptake by control (0% FCS) cultures ($P<.01$); except the uptake by cell cultures, cultured with 10% FCS, for a 1 hour incubation period, which was also significantly different from the uptake of isotope uptake by control cultures, ($0.05 > P > 0.01$). The uptake of isotope by cell cultures, cultured with 15% FCS, was inhibited in cultures incubated for 1, 2 and 6 hours; and was significantly different from the uptake of isotope by control

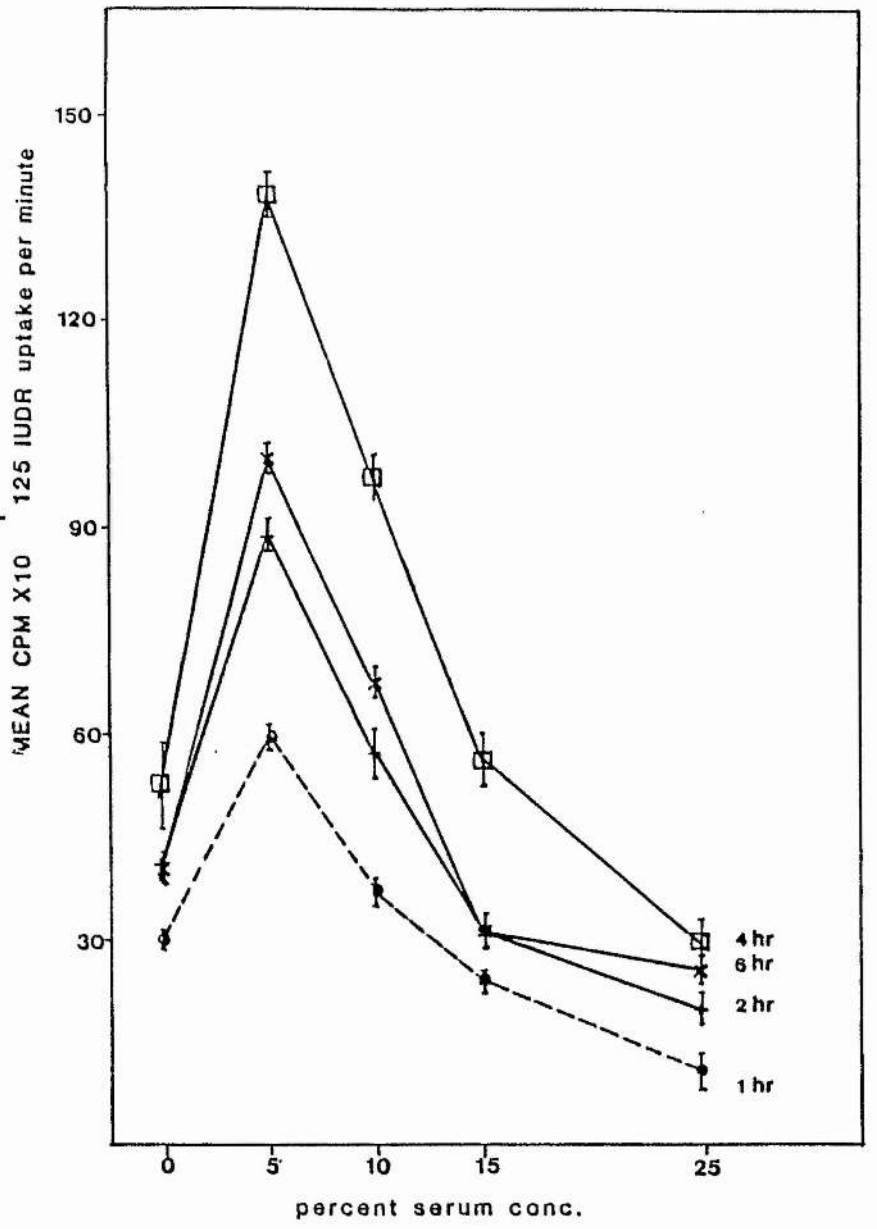


FIGURE 1.2.2.3 : The uptake of 125IUDR by thymocyte cultures, cultured with different concentrations of FCS-HI for different periods of incubation time .

Table 1.2.2.3: The mean uptake of 125IUDR by thymocytes, expressed as counts per minute (cpm) and as percent inhibition cultured in different concentractions of FCS for different incubation periods. The ± standard error values are included.

Percent FCS concentration	Incubation period (hours) and cpm (±S.E.)			
	(1)	(2)	(4)	(6)
0%	302 ± 12	412 ± 8	530 ± 62	398 ± 17
5%	596 ± 18	889 ± 26	1381 ± 39	1002 ± 20
10%	370 ± 20	575 ± 38	977 ± 30	680 ± 16
15%	240 ± 15	307 ± 21	565 ± 39	312 ± 34
25%	110 ± 25	205 ± 30	297 ± 32	259 ± 18
	Percent inhibition			
5%	+ 97.20%	+ 113.19%	+ 160.54%	+ 151.48%
10%	+ 22.47%	+ 37.97%	+ 84.28%	+ 70.64%
15%	- 20.44%	- 26.16%	- 6.61%	- 21.66%
25%	- 63.46%	- 50.77%	- 43.99%	- 34.90%

\+ = no inhibition (stimulation) of IUDR uptake.
\- = inhibition

cultures, for the 1 and 2 hour cultures, (0.05 >P> 0.01) and (P<.01) respectively; while in the 6 hour cultures, the inhibition was significantly different (0.05 > P >.01) from the uptake of isotope by control cultures. At four hours of incubation, there was a minimal change in isotope uptake by cultures (P > .05) which was not significantly different from the uptake by control cultures. At 25% FCS for all incubation periods, the change in isotope uptake by cultures was significantly different from the uptake by control cultures (0% FCS), (P < .01); except at 4 hours incubation, the uptake of isotope by cultures was significantly different , at (0.05 > P > .01). The cell viability was assessed as described earlier. Two incubation periods were chosen, the 4 and 6 hours, and in both these periods, the cell viabilities of cultures, cultured with 25% FCS, were not significantly different (P >.05), from their counterpart cultures, cultured with 0% or 5% FCS.

As described above, (section 3.1.2.1), the inhibition of isotope uptake by FCS was assessed at two FCS (56 and 66 ^{0}C) heat treatments. The increase in the heat treatment (66^{0}C) did not eliminate the inhibitory effects of FCS on isotope uptake (as compared to 56^{0}C) by cell cultures. The uptake of 125IUDR by the respective cell cultures described above, (section 3.1.2.2) was assessed in terms of the increase of the concentration of FCS in the culture environments; to know whether higher FCS concentrations exert more inhibition on the uptake of isotope by the respective cultures. The results have demonstrated that the inhibition of isotope uptake varied directly with the concentration of FCS. The use of three different cell lines for culture, was undertaken to assess whether the interaction of FCS with each culture type (kinetics of isotope uptake inhibition) was variable. The results

have demonstrated that FCS interacted differently with each culture, in terms of serum concentration and incubation time.

All cultures shared a common feature, which was the increase in isotope uptake inhibition as the concentration of FCS per culture was increased. However, the kinetics of isotope uptake inhibition varied with each culture type in terms of the incubation period and the concentration of FCS per culture. For plasmacytoma, the inhibitory effects of FCS were reduced with increasing incubation time, for all concentrations of FCS. Mammary adenocarcinoma reacted differently to FCS, the general pattern of isotope uptake was stimulted rather than inhibited, at short incubation incubation periods, but was the uptake of isotope was inhibited at longer incubation periods. For thymocytes there was a constant pattern of stimulation of uptake at 5 and 10% FCS concentrations, and at 15% FCS there was a general constant pattern of uptake inhibition (versus incubation period), while at 25% FCS the inhibition of isotope uptake was reduced with increasing incubation time, (i.e. similar to the uptake of isotope by plasmacytoma cultures at 25% FCS).

3.1.3: The uptake of tritiated thymidine by plasmacytoma cultures, cultured at different cell concentrations per culture well, in several concentrations of FCS.

Experimental procedures:

Plasmacytoma cells were cultured in microtitre culture wells at a concentration of 5×10^4, 1×10^5, and 5×10^5 cells per well as described earlier, and were incubated for 1 hour as described previously. Cultures were cultured with the following FCS-HI concentrations: 0%, 5%, 10%, 15% and 25%; each well received 0.2 uci of 3H-Tdr per a 200 ul cell suspension per well. Each culture was replicated 5 times. The uptake of isotope by cultures was assessed as described earlier.

Results:

The uptake of 3H-Tdr by cell cultures has decreased as the FCS concentration per culture increased, (Figure/Table 1.3). The highest mean cpm was for cells cultured without FCS, while the lowest mean cpm was for cells cultured with 25% FCS. The percentage of 3H-Tdr uptake inhibition has increased, as the FCS concentration increased in each cell dose group. The difference between the mean cpm of cultures , cultured without FCS, and cultures, cultured with the other four concentrations of FCS, in the 1×10^5 cell culture group, were significant. The assessment of the significance of the these results was based on an analysis of variance, and Dunnett's procedure;(Anovar; F.ratio=127.14; significant, $P<.01$), Dunnett's test (2 tail, $P = 0.1$). Similarly, for the 5×10^5 cells/well group, all the differences between the respective mean cpm of cultures, cultured with the respective FCS

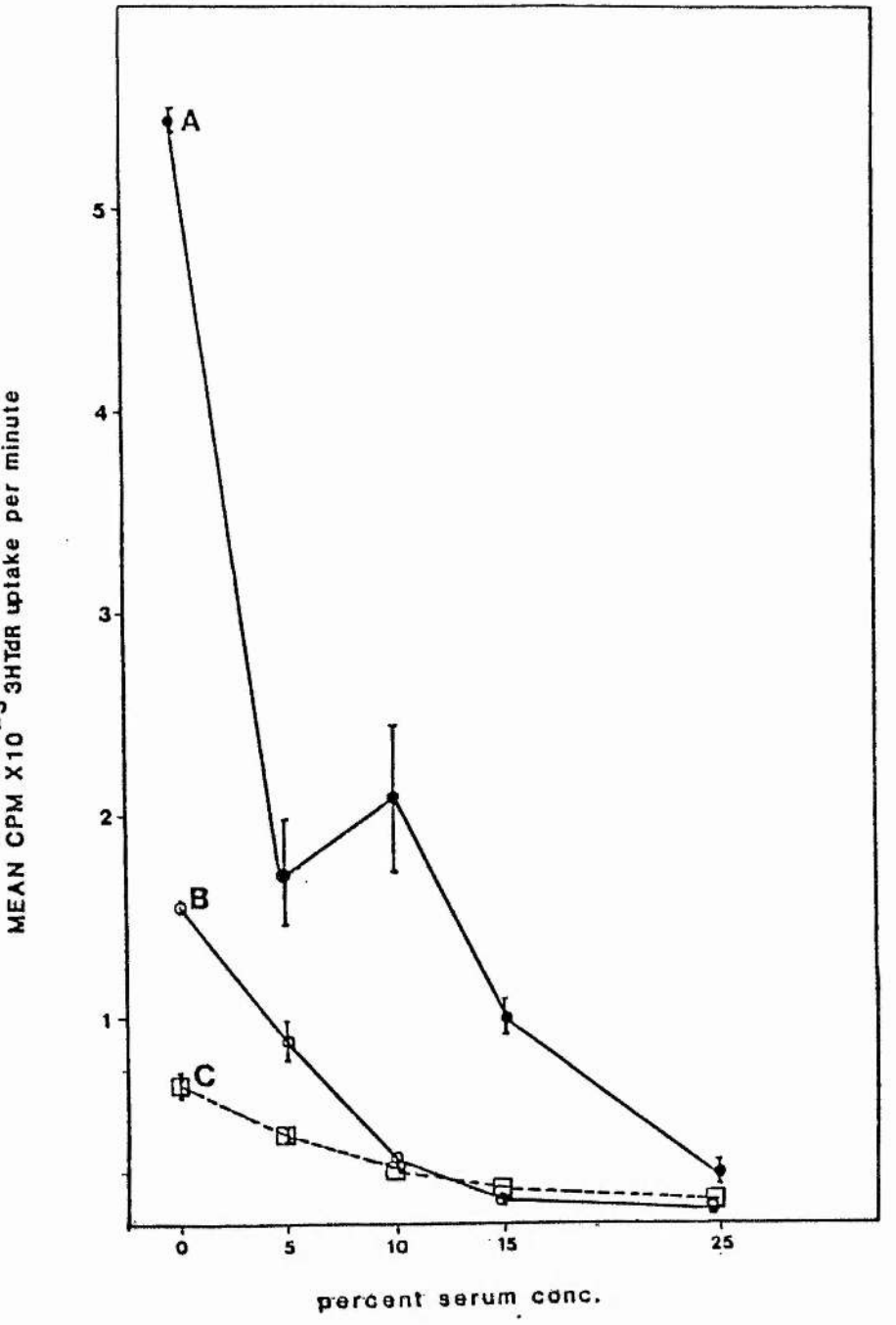


FIGURE 1.3: The uptake of 3H-Tdr by plasmacytoma cultures, cultured for 4 hours with different concentrations of FCS-HI.
A: 5×10^5 cells/culture, B: 1×10^5 cells/culture, C: 5×10^4 cells/culture.

Table 1.3 ; The mean uptake (cpm) of ^{3}H-Tdr by plasmacytoma cells cultured for 1 hour at 5 x 10^{4}, 10^{5}, and 5 x 10^{5} cells per well, in the presence of 0%, 5%, 10%, 15% and 25% FCS. The mean uptake of isotope is also expressed as percent inhibiton. The ± S.E. values are included.

	Cell dose/well		
Serum concentration	5 x 10^{4}	1 x 10^{5}	5 x 10^{5}
	mean (cpm) ± S.E.		
Medium (0%)	688 ± 46	1547 ± 37	5444 ± 91
5%	435 ± 463	916 ± 95	1704 ± 231
10%	264 ± 49	314 ± 41	2108 ± 323
15%	181 ± 34	127 ± 26	960 ± 102
25%	122 ± 13	77 ± 12	268 ± 29
	Percent inhibition ± S.E.		
Serum concentration	5 x 10^{4}	1 x 10^{5}	5 x 10^{5}
5%	36.8 ± 6.7	40.8 ± 6.2	68.7 ± 4.26
10%	61.52 ± 7.25	79.67 ± 2.68	61.27 ± 5.96
15%	73.67 ± 5	91.73 ± 2	82.35 ± 1.6
25%	82.25 ± 2	94.96 ± 0	95.07 ± 0

concentrations, were significant; (Anovar; F.ratio = 150.16; significant, P < .01) Dunnett's test (2 tail, P = 0.1).

The cell viabilities of cell cultures, cultured for 1 hour with 10% and 25% FCS for the 1 x 10^5 and the 5 x 10^5 cells/well groups, were not significantly different from their respective control cultures which were cultured without FCS, (t-test, P>.01) .

As described above, (section 3.1.3) the inhibition of isotope uptake by FCS on plasmacytoma cultures, using another isotope (3H-Tdr) was assessed. The results described above, have shown that the inhibition of isotope uptake was not due to the use of another isotope such as 125IUDR (which may not be as efficiently utilized by the DNA enzymes system as 3H-Tdr) as the uptake of 3H-Tdr was subjected to a similar pattern of inhibition by FCS.

3.1.4: The effects of different batches of FCS on the uptake of 125IUDR by plasmacytoma cultures.

Experimental procedures:

A plasmacytoma cell suspension was prepared and dispensed into microtitre wells at a cell dose of 1 x 10^5 cells per well. The cells were cultured for 4 hours with three different batches of FCS-HI, at 37 ^{o}C, 5% CO_2. Cell cultures were cultured with 10% and 25% serum. Each culture received 0.2 uci of 125IUDR, and was replicated 5 times. The uptake of isotope by cell cultures was assessed as described previously.

Table 1.4 : The effects of three different batches of FCS (A, B and C) on the uptake of 125IUDR by plasmacytoma cells, cultured in 10% and 25% serum concentration. The values are expressed as mean cpm and as percent inhibition. ± S.E. is included.

Batch	Mean cpm ± S.E.		
	FCS concentration		without FCS
	10%	25%	
A	5896 ± 267	1569 ± 92	8279 ± 560
B	4149 ± 418	1311 ± 100	8676 ± 227
C	6086 ± 134	2075 ± 104	12269 ± 393
	Percent inhibition ± S.E.		
A	28.77 ± 3.23	81.04 ± 0	
B	52.17 ± 5	84.88 ± 1	
C	50.39 ± 1	83.08 ± 1	

Results:

Two batches of FCS-HI (B and C) were more inhibitory than batch "A" on the uptake of isotope by cultures, ($P < .01$), the difference in inhibition was approximately twofold at 10% FCS (Table 1.4). The isotope uptake by cultures was similar at 25% serum concentration, without significant differences ($P > .05$). The effects of FCS-HI batches "B and C" on the uptake of isotope by cultures, cultured with 10% serum, were similar, ($P > .05$).

As described above, (section 3.1.4) different batches of FCS were examined in terms of their effects on the uptake of isotope by cell cultures. The results have shown that different FCS batches exhibit either different or similar inhibitory patterns depending on their concentration in the culture environment.

3.1.5: The specificity of the uptake of 125IUDR by DNA of plasmacytoma cultures.

Experimental procedures:

3.1.5.1.1: A plasmacytoma suspension was prepared as described previously, and was dispensed into sterile culture tubes; at a dose of 0.5×10^6 cells/200 ul per culture tube. Cell cultures were cultured in the following FCS concentrations 0%, 5%, 10%, 15% and 25% . Each culture received 0.2 uci of 125IUDR, and was replicated 5 times. Cultures were incubated at 37 °C, 5% CO_2 for 4 hours as described earlier. Acid insoluble DNA was thereafter extracted in NaOH and TCA as described earlier. The amount of 125IUDR activity (mean cpm) in the DNA extracts was assessed as

described previously.

Results:

The amount of radioactivity recovered in the acid insoluble fraction of DNA, has decreased as the serum concentration increased stepwise from 0% to 5%, 10%, 15% and 25% FCS per culture, (Figure/Table 1.5.1.1). The highest mean cpm (1649) was present in DNA extracts of cultures, cultured without FCS; while the lowest mean cpm (151) was present in DNA extracts of cultures, cultured with 25% FCS. The amount of radioactivity recovered in the soluble fraction was highest in extracts of cells cultured without serum and lowest in extracts of cultures, cultured with 25% FCS. All the values (mean cpm) of cells cultured in their respective serum concentrations were significantly ($P < .01$) different from the mean cpm of control cultures (without FCS). An analysis of variance on the mean cpm of extracts of the insoluble fraction, i.e., , using extracts of cells cultured with 0%, 5%, 10%, 15% and 25% FCS, was significant; (Anovar F.ratio = 10.45, $P<.01$). The comparisons between the respective means using Dunnett's test procedure (2 tail, $P = .01$); have shown that DNA insoluble extracts obtained from cells cultured with 25% FCS have a significantly different mean cpm from the mean cpm of extracts of cultures, cultured with 5% FCS and 0% FCS, but not different from the mean cpm of extracts of cells cultured with 10% or 15% FCS.

Experimental procedures:

3.1.5.1.2: A cell suspension of plasmacytoma cells was prepared and dispensed into microtitre wells at 1×10^5 per culture well. Each cell dose was cultured with and without 10% FCS-HI in

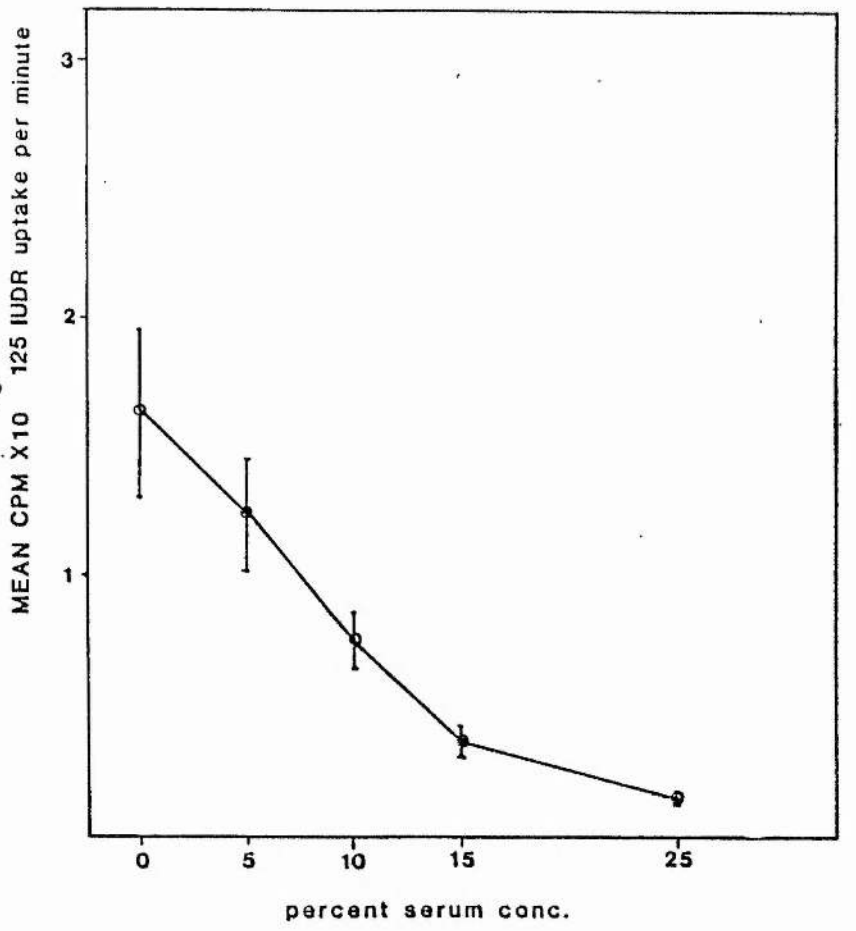


FIGURE 1.5.1.1: The uptake of 125 IUDR by plasmacytoma cultures, cultured with different concentrations of FCS-HI for 4 hours. The effect of serum concentration per culture on the amount of radioactivity of 125IUDR recovered in the acid insoluble fraction of DNA .

Table 1.5.1.1 The uptake of 125IUDR uptake expressed as mean cpm and percent inhibition recovered in the acid insoluble fraction of DNA, from plasmacytoma cells cultured in the presence of medium (0%), 5%, 10%, 15% and 25% (FCS.HI) for 4 hours. The ± S.E. values are included.

Serum concentration	Insoluble fraction mean cpm ± S.E.	Acid soluble fraction mean cpm ± S.E.
0% (Medium)	1649 ± 351	746 ± 34
5%	1253 ± 216	523 ± 38
10%	761 ± 99	287 ± 16
15%	358 ± 63	287 ± 31
25%	151 ± 47	216 ± 8
Serum concentration	Percent inhibition ± S.E.	
0%	0.0	0.0.
5%	24.03 ± 13.18	29.85 ± 4.67
10%	53.86 ± 6.02	61.53 ± 2.22
15%	78.24 ± 3.83	61.46 ± 4.24
25%	90.83 ± 1.29	71.03 ± 1

the following molar concentrations of hydroxyurea : 0, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}. The final volume per well was 200 ul, each cell dose received 0.2 uci 125IUDR, and was replicated 5 times. Cultures were incubated for 4 hours, as described previously. The uptake of isotope by cultures was described earlier.

Results:

The uptake of 125IUDR has decreased as the concentration of hydroxyurea increased, for both cell concentrations, cultured with or without 10% FCS, (Figure/Table 1.5.1.2). The lowest inhibition of 125IUDR uptake by cell cultures was at concentration of 10^{-6} moles hydroxyurea, with almost complete inhibition being achieved at 10^{-3} moles hydroxyurea, with a slight increase in inhibition at 10^{-1} moles hydroxyurea. The mean cpm of cultures, cultured with FCS, and 10^{-6} moles hydroxyurea, was significantly different from the mean cpm of control culture ($0.05 > P > .01$), similarly the mean cpm of cultures cultured with concentrations of hydroxyurea above 10^{-6} moles, were also significantly different , ($P < .01$). For cultures, cultured without FCS, the mean cpm of cultures, cultured in concentrations of hydroxyurea of 10^{-4} moles to 10^{-1} moles, were also significantly different from the mean cpm ($P<.01$) of control cultures.

Experimental procedures:

3.1.5.2: A plasmacytoma cell suspension was prepared and dispensed into microtitre wells at 1×10^{5} cells per well. Cells were cultured with 10% FCS, 5% FCS and without FCS , in the presence of the following molar concentrations of thymidine: 1×10^{-7}, 5×10^{-7}, 1×10^{-6}, 5×10^{-6}, 1×10^{-5} and 1×10^{-4}. Cell cultures

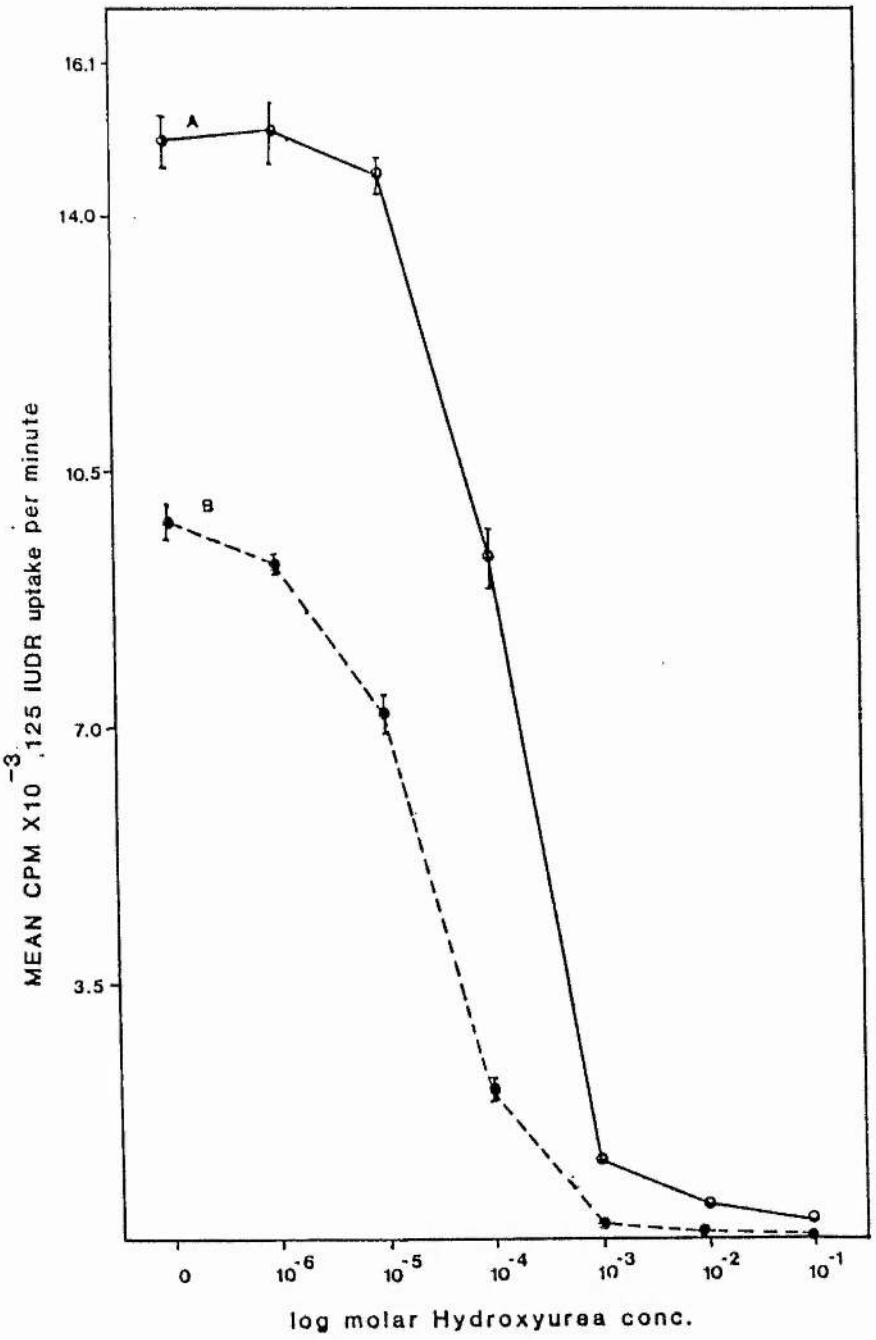


FIGURE 1.5.1.2: The uptake of 125IUDR by plasmacytoma cultures, cultured for 4 hours with different concentrations of hydroxyurea.
A: without FCS, B: with 10% FCS .

Table 1.5.1.2; The mean uptake of 125IUDR by plasmacytoma cells, expressed as counts per minute (cpm) cultured for 4 hours with 10% FCS and without FCs, in several concentrations of Hydroxyurea. ± Standard error values are included.

Molar concentration	with 10% FCS		without 10% FCS	
Hydroxyurea/well	mean cpm ± S.E. 1×10^5 S.E.	Percent inhibition ± S.E.	mean cpm ± S.E. 1×10^5 S.E.	percent inhibition ± S.E.
0	9835 ± 207	0.0	15052 ± 316	0.0
10^{-6}	9219 ± 86	6.26 ± 4.85	15199 ± 301	+ 0.97 ± 0.01
10^{-5}	7265 ± 158	26.13 ± 1.6	14565 ± 255	3.23 ± 1.69
10^{-4}	2044 ± 108	79.21 ± 1	9314 ± 434	38.12 ± 2.89
10^{-3}	242 ± 94	97.52 ± 0	1071 ± 44	92.88 ± 0
10^{-2}	95 ± 67	99.03 ± 0	425 ± 15	97.17 ± 0
10^{-1}	74 ± 33	99.24 ± 0	203 ± 8	98.64 ± 0

+ = stimulation of uptake.

were incubated for 4 hours as described previously. Each culture with its respective thymidine concentration was replicated in 5 wells, with 10% FCS, 5% FCS and without FCS. Each culture received 0.2 uci of 125IUDR. The final volume per well was 200 ul. The uptake of 125IUDR by cultures was assessed as described earlier. The amount of thymidine in FCS was assessed in two ways :

1. The difference in isotope uptake (mean cpm), between control cultures without FCS (0% FCS), and control cultures with FCS (5% and 10% FCS), was determined as a percent inhibition of isotope uptake :

[A - B] / [A] = % inhibition

A = mean cpm of 0% FCS cultures

B = mean cpm of 5% or 10% FCS cultures

In Figure 1.5.2 , Curve A = cultures without FCS; Curve B = cultures with 5% FCS and Curve C = cultures with 10% FCS.

The estimated percent inhibition of isotope uptake by 5% and 10% FCS, were related to Curve A, to estimate the equivalent amount of thymidine per culture that may produce the respective inhibitions of isotope uptake by 5 or 10 percent FCS.

2. The shifts in Curve B, and Curve C, relative to Curve A, in terms of the molar concentration of thymidine per culture, at the 50% inhibition value of isotope uptake of Curve A, at the linear segments of the curves.

Results:

The uptake of 125IUDR was represented by 3 curves, (Figure/Table 1.5.2) curves A, B, and C, represented the uptake of isotope by cultures, cultured without FCS, and with 5% and 10% FCS respectively. As the molar concentration of thymidine per culture, has increased, the uptake of isotope by respective cultures, decreased. The highest inhibition of isotope uptake in the respective cultures, (90 to 99 percent), was achieved by a concentration of thymidine between 5×10^{-6} moles to 1×10^{-4} moles per culture. The lowest inhibition of isotope uptake by the respective cultures, was achieved by a thymidine concentration of 1×10^{-7} moles per culture.

The amount of isotope uptake by cell cultures, cultured with 5% and 10% FCS (control cell cultures: without thymidine), compared to cultures, that were cultured without FCS (control cell cultures: without thymidine), expressed as percent inhibition of isotope uptake was equal to: 9.42% and 29.75% respectively. The amount of thymidine per culture, that produced 9.42% and 29.75% inhibition of isotope uptake by cultures, as determined in terms of Curve A, was equal to: 1.42×10^{-7} moles and 3×10^{-7} moles respectively.

The molar concentrations of thymidine per culture, which produced 50% inhibition of isotope uptake by the respective cultures, as represented in Curves A, B, and C, were 6.6×10^{-7}, 5.1×10^{-7}, and 3.2×10^{-7} moles respectively. Thus the amount of thymidine in 5% FCS was equal to: $[(6.6 \times 10^{-7}) - (5.1 \times 10^{-7})]$, 1.5×10^{-7} moles. Similarly, the amount of thymidine in 10% FCS was equal to: $[(6.6 \times 10^{-7}) - (3.2 \times 10^{-7})]$, 3.2×10^{-7} moles. The 50% inhibition of isotope uptake by cell cultures, for all 3 curves

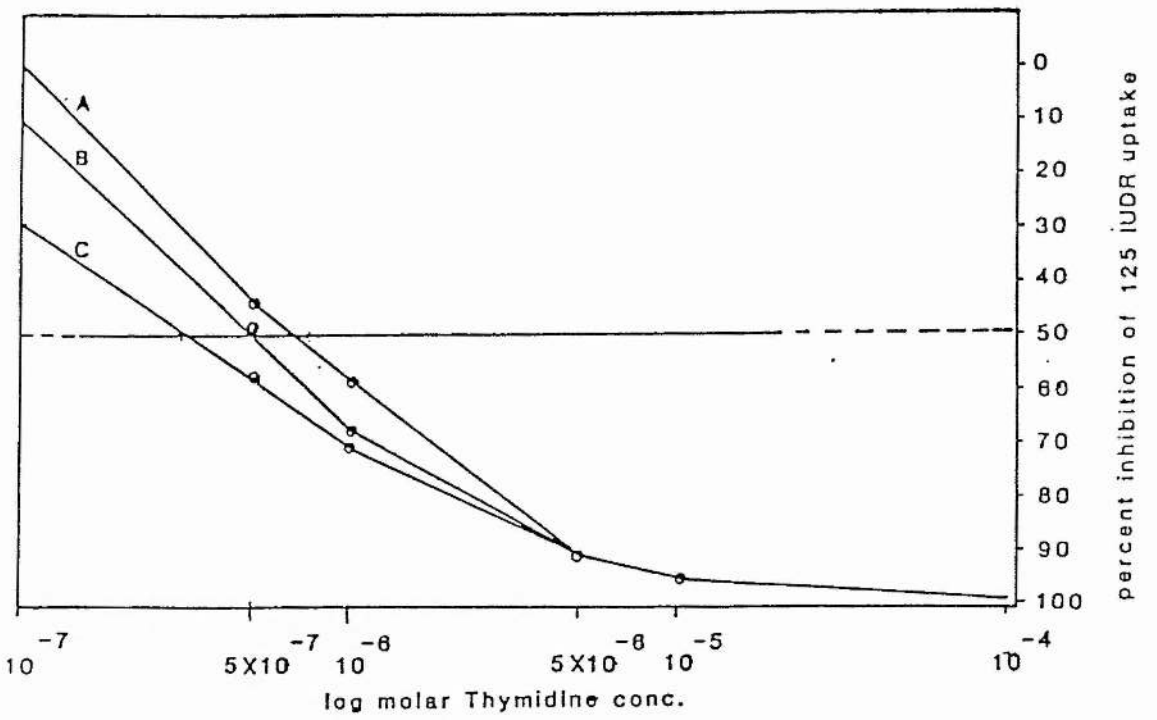


FIGURE 1.52 The uptake of 125IUDR by plasmacytoma cultures, cultured with different concentrations of thymidine for 4 hours. A: without FCS, B: with 5% FCS-HI, C: with 10% FCS-HI .

Table 1.5.2: The effect of increasing the concentration of added thymidine to plasmacytoma cell cultures on the uptake of 125IUDR by plasmacytoma cultures. The uptake of 125IUDR is described in terms of counts per minute (cpm) and as percent inhibition with ± S.E. included.

	Without serum (medium only)		5% FCS		10% FCS	
Thymidine concentration	Mean cpm ± S.E.	Percent inhibition	Mean cpm ± S.E.	Percent inhibition	Mean cpm ± S.E.	Percent inhibition
0 (controls)	8495 ± 181	0.00	7695 ± 66	9.42 ± 0	5968 ± 332	29.75 ± 4
1×10^{-7}M	8151 ± 387	4.05 ± 4.63	7645 ± 151	10.00 ± 1.68	5992 ± 528	29.47 ± 6.2
5×10^{-7}M	4782 ± 564	43.7 ± 4.4	4480 ± 156	47.26 ± 1.84	3653 ± 165	56.99 ± 1.77
1×10^{-6}M	3491 ± 2	58.9 ± 0	2658 ± 161	68.71 ± 1.9	2503 ± 88	70.53 ± 1
5×10^{-6}M	817 ± 107	90.38 ± 1	832 ± 53	90.19 ± 0	814 ± 30	90.40 ± 0
1×10^{-5}M	477 ± 5	94.38 ± 0	441 ± 18	94.80 ± 0	485 ± 19	94.29 ± 0
1×10^{-4}M	132 ± 12	98.44 ± 0	122 ± 4	98.55 ± 0	125 ± 8	98.52 ± 0

was between 1 x 10^{-7} and 1 x 10^{-6} moles. Thus the amount of thymidine estimated from Curves A, B and C by the 2 methods as described in experimental procedures were similar. In the first method 5% FCS contained 1.42 x 10^{-7} moles, and 10% FCS contained 3 x 10^{-7} moles. In the second method 5% FCS contained 1.5 x 10^{-7} moles and 10% FCS contained 3.4 x 10^{-7} moles.

As described above, (section 3.1.5) the inhibitory effects of FCS were assessed in terms of the specificity of the uptake of isotope by DNA of cell cultures. The results above, have shown that the inhibitory effects of FCS on isotope uptake were specific, in the sense, that the amount of 125IUDR available in the DNA extracts has decreased as the concentration of FCS was increased in the culture environment. The pattern of isotope uptake by DNA was corroborated by the use of hydroxyurea, a phase specific (S phase) inhibitory substance, to demonstrate the pattern of isotope uptake inhibition by such a substance. The uptake of isotope was also assessed in terms of the inhibitory effects of thymidine, (as FCS may contain a vareity of nucleotides) to show the pattern of the inhibitory effects of thymidine on the uptake of isotope by cell cultures, as thymidine may compete with the labelled nucleotide, or block the uptake, depending on its concentration in the culture environment.

3.1.6: The effects of serum type, serum subfraction, cell culture density and incubation period on the uptake of 125 IUDR by cell cultures.

Experimental procedures:

3.1.6.1: Cell suspensions of plasmacytoma and mouse thymocytes were prepared, and two concentrations of each cell type were dispensed into microtitre wells: 5×10^4 cells/well and 1×10^5 cells/well. Cell cultures of plasmacytoma were cultured in different concentrations 0%, 5%, 10%, 15%, and 25% of FCS-HI and BSA-HI respectively; while mouse thymocytes were cultured in FCS-HI. The final volume per culture well was 200 ul; cultures received 0.2 uci 125IUDR, and were replicated 5 times. Cell cultures were incubated at 37 ^{0}C, 5% CO_2, as described previously. The uptake of isotope by cultures was assessed as described previously.

Results:

The mean cpm of plasmacytoma cultures has decreased as the concentration of FCS per culture increased, (Figure/Table 1.6.1.1). In the 5×10^4 cell culture group, 5% FCS has stimulated the uptake of isotope by respective cultures; while 10% FCS has relatively inhibited the uptake of isotope by respective cultures, but was not significantly different ($P > .05$), from the uptake of isotope by control cultures. FCS at 15% and 25% per culture has inhibited the uptake of isotope by respective cultures, and the respective isotope uptake was significantly different (15% FCS: $.05 > P > .01$; 25% FCS: $P < 0.01$) from the uptake of isotope by control cultures. In the 1×10^5 cell culture group, all concentrations of FCS

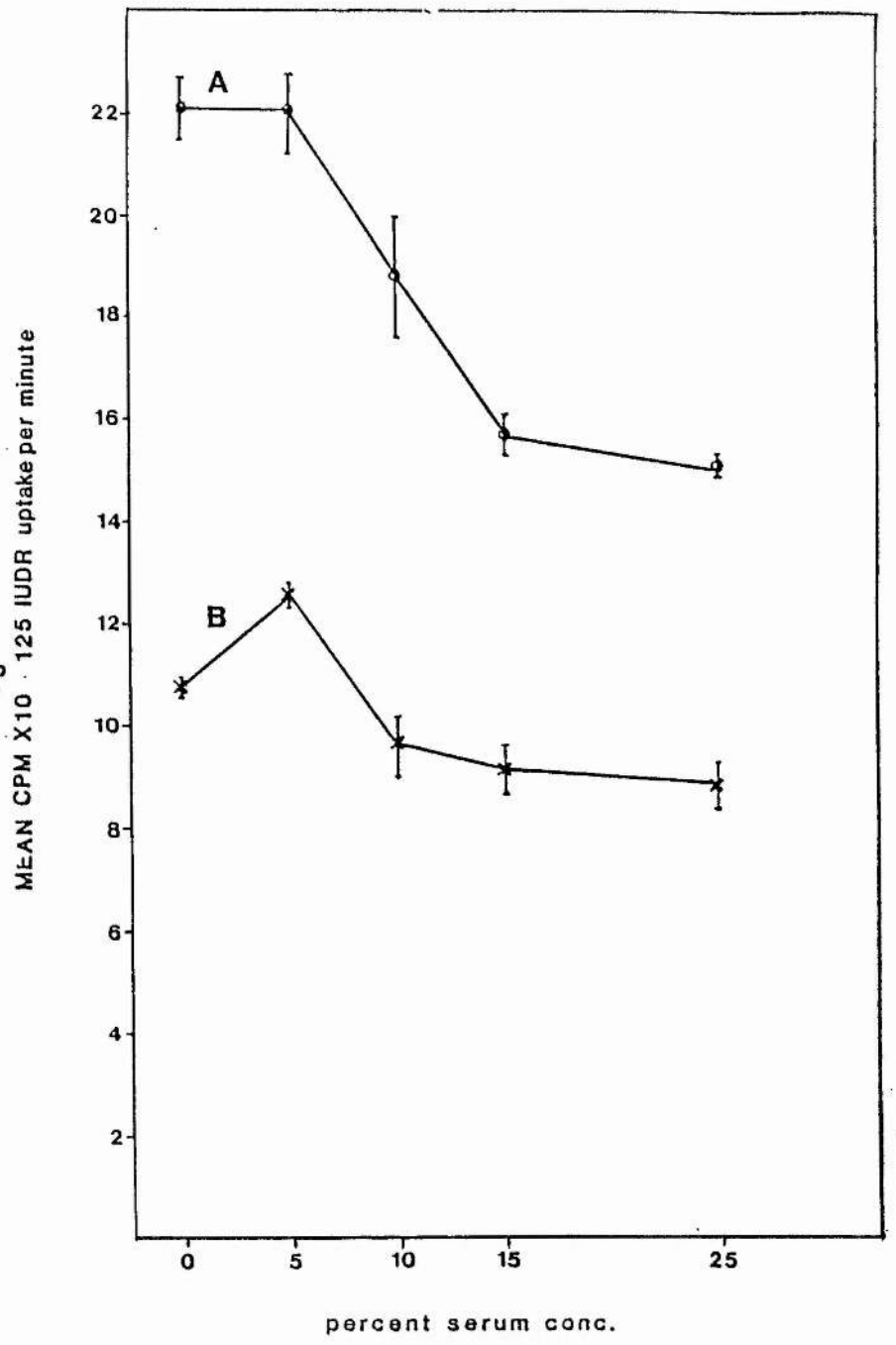


FIGURE 1.6.1.1: The uptake of 125 IUDR by plasmacytoma cultures, cultured with FCS-HI for 4 hours.
A: 1×10^5 cells/culture, B: 5×10^4 cells/culture .

Table 1.6.1.1: The mean uptake of 125IUDR uptake (cpm) by 2 cell culture concentrations of plasmacytoma 5×10^4 and 1×10^5 cells/well cultured in 0%, 5%, 10%, 15% and 25% FCS, incubated for 4 hours. ± Standard error values are included. The percent inhibition of isotope uptake is included.

	(FCS-HI Concentration Percent) and mean cpm ± S.E.				
Cell concentration	(0%)	(5%)	(10%)	(15%)	(25%)
5×10^4	10759 ± 130	12605 ± 112	9724 ± 514	9146 ± 470	8903 ± 477
1×10^5	22124 ± 642	22098 ± 782	18822 ± 782	15734 ± 420	15097 ± 217
	Percent inhibition ± S.E.				
5×10^4		+17.15 ± 1	9.61 ± 4.78	14.99 ± 4.37	17.24 ± 4.43
1×10^5		0.11 ± 3.42	14.92 ± 5.28	28.88 ± 2	31.76 ± 1

+ = stimulation of uptake.

inhibited the uptake of isotope by respective cultures. However, the uptake of isotope by cultures cultured with 5% FCS, was not significantly different ($P > .05$), from the uptake of isotope by control cultures. The uptake of isotope by cultures, cultured with 10, 15, or 25 percent FCS, was significantly different (10% FCS: $.05 > P > .01$; 15% and 25% FCS: $P < 0.01$).

The percentage of isotope uptake inhibition has increased in the 1×10^5 cell culture group relative to the 5×10^4 cell group, thus at 10, 15 and 25 percent FCS , there was an increase of 55, 92, and 84 percent inhibition respectively, in the 1×10^5 cell culture group relative to the 5×10^4 cell culture group. At 10% FCS the change was not significant ($P > .05$) while at 15-25% FCS the change was significant ($0.05 > P > .01$).

The mean cpm of plasmacytoma cultures has decreased as the concentration of BSA per culture increased, (Figure/Table 1.6.1.2). The inhibition of isotope uptake in the 5×10^4 and the 1×10^5 cell culture groups was generally similar at 5%, 10%, 15% and 25% BSA per culture. The mean cpm of cultures was significantly different ($P < .01$), from the mean cpm of their respective control cultures. The percent change in isotope uptake inhibition in the 1×10^5 cell culture group at the respective BSA concentrations relative to the 5×10^4 cell culture group was about 7.5% for 10% BSA, none at 15% BSA) and about 3% at 25% BSA, all of which were not significant ($P > .05$).

The mean cpm of thymocyte cultures has decreased, as the concentration of FCS per culture increased, (Figure/Table 1.6.1.3). For both cell culture groups, cultured with 5% FCS, the percent change in isotope uptake was not significantly different ($P > .05$), from the uptake of isotope by control cultures; while the uptake of

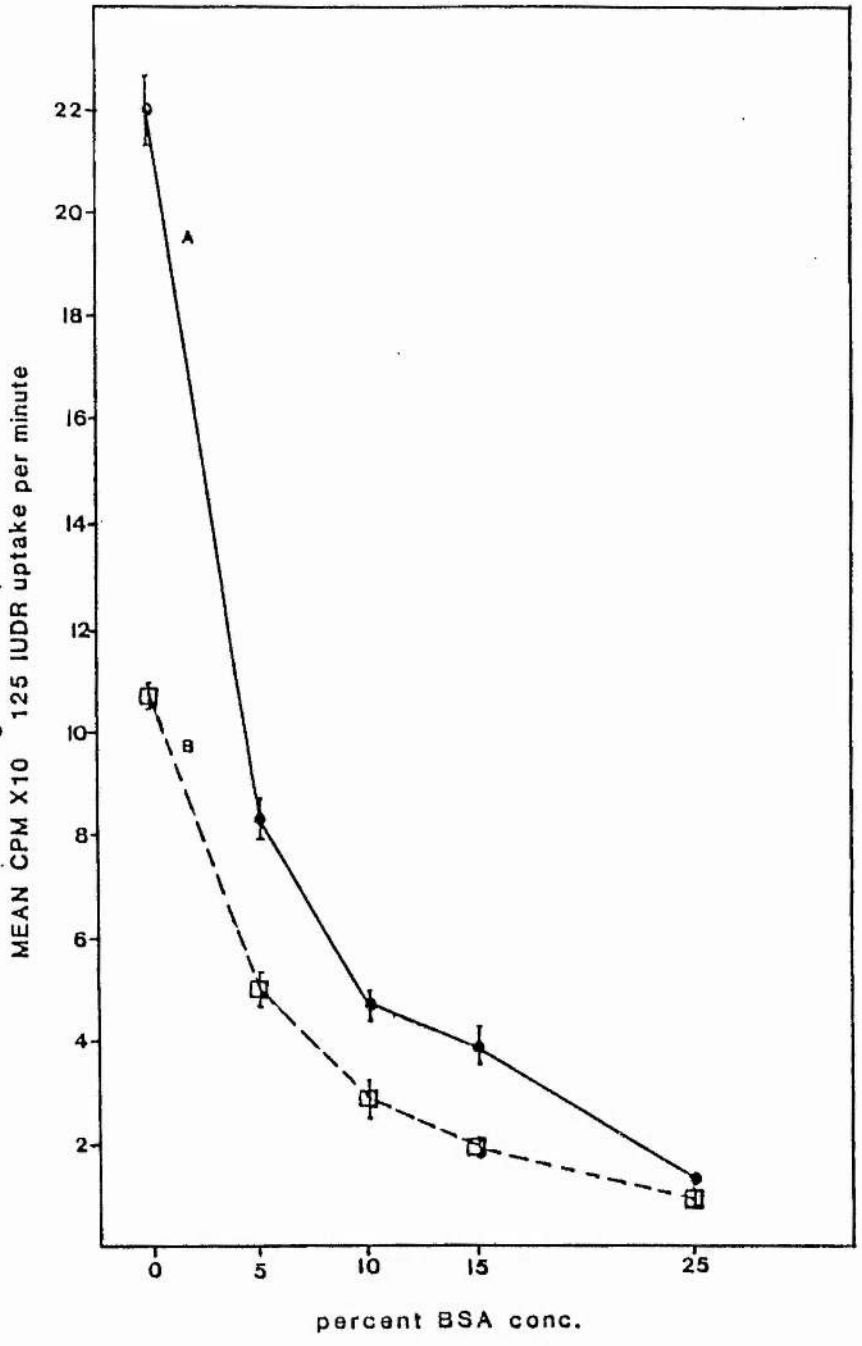


FIGURE 1.6.1.2 The uptake of 125IUDR by plasmacytoma cultures, cultured with BSA for 4 hours.
A: 1 x 10^5 cells/culture, B: 5 x 10^4 cells/culture .

Table 1.6.1.2: The mean uptake of 125IUDR uptake (cpm) by 2 cell culture concentrations of plasmacytoma, 5×10^4 and 1×10^5 cells/well, cultured in 0%, 5%, 10%, 15% and 25% BSA, incubated for 4 hours. ± Standard error values are included. The percent inhibition of isotope uptake is included.

	(BSA concentration percent) and mean cpm ± S.E.				
Cell concentration	(0%)	(5%)	(10%)	(15%)	(25%)
5×10^4	10759 ± 130	5058 ± 226	2907 ± 258.4	1932 ± 111	925 ± 93
1×10^5	22124 ± 642	8328 ± 383	4763 ± 220	3984 ± 376	1270 ± 29
	Percent inhibition ± S.E.				
5×10^4	-	52.97 ± 2.1	73 ± 2.4	82.04 ± 1	91.29 ± 0
1×10^5	-	62.23 ± 1.7	78.47 ± 1	81.99 ± 1.7	94.25 ± 0

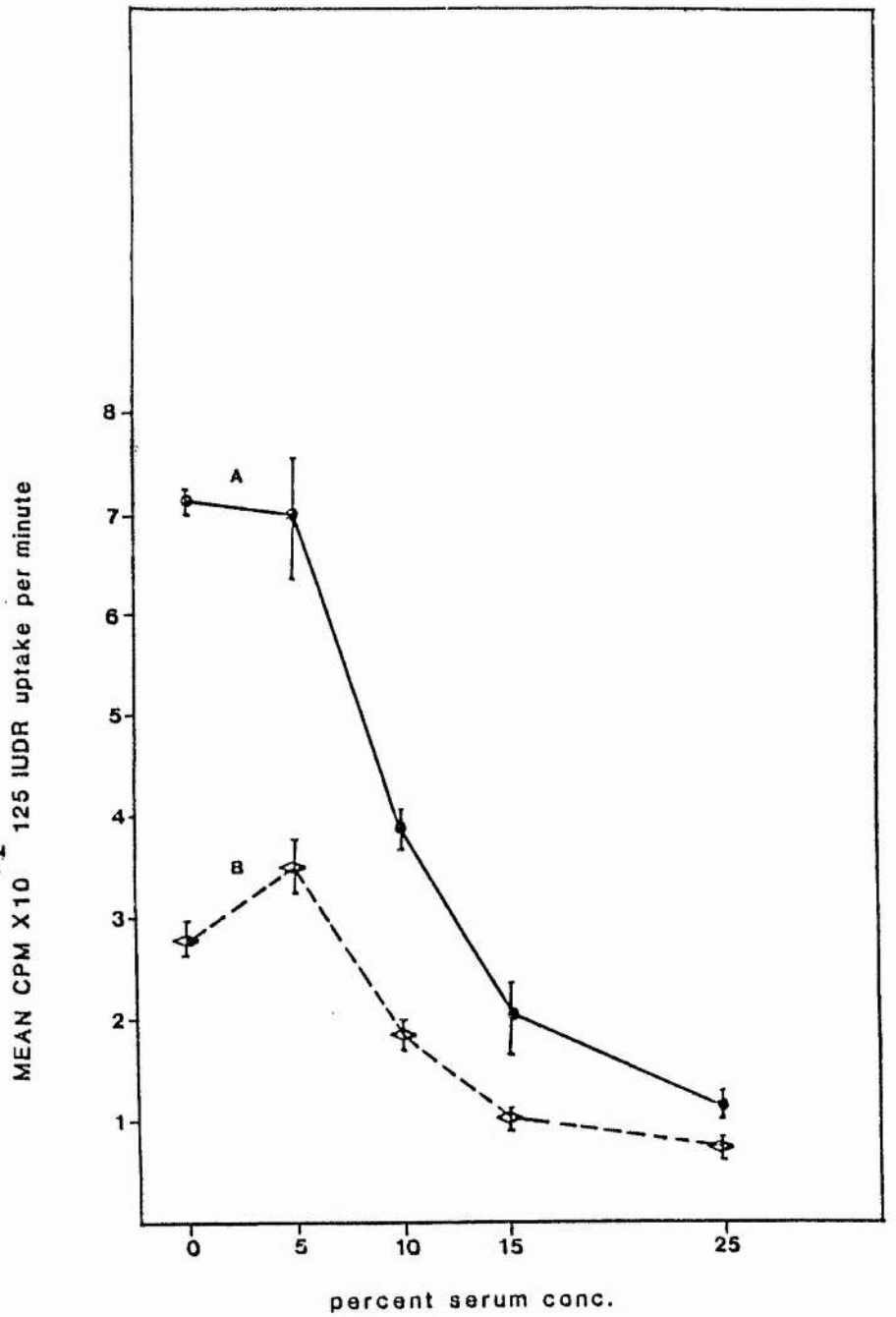


FIGURE 1.6.1.3 The uptake of 125IUDR by mouse thymocyte cultures, cultured with FCS-HI for 4 hours.
A: 1 x 10^5 cells/culture, B: 5 x 10^4 cells/culture .

Table 1.6-1.3: The mean uptake of 125IUDR uptake (cpm) and percent inhibition by 2 cell concentrations of mouse thymocytes; 5 x 10^4 and 1 x 10^5 cells/well, cultured in 0%, 5%, 10%, 15% and 25% FCS; incubated for 4 hours. ± Standard error values are included. The percent inhibition of isotope uptake is included.

	(FCS-HI concentration percent) and mean cpm				
Cell Concentration	(0%)	(5%)	(10%)	(15%)	(25%)
5 x 10^4	281 ± 14	351 ± 27	186 ± 12	103 ± 8	74 ± 9
1 x 10^5	715 ± 11	701 ± 56	387 ± 18	206 ± 38	113 ± 14
	Percent Inhibition				
5 x 10^4		+24.94 ± 9.95	33.84 ± 4.6	63.34 ± 3.2	73.63 ± 3.4
1 x 10^5		1.98 ± 7.88	45.87 ± 2.62	71.19 ± 5.34	84.19 ± 2

+ = stimulation of uptake.

isotope by cultures, cultured with 10%, 15% and 25% FCS was significantly different (P <.01). The percentage of isotope uptake inhibition in the 1 x 10^5 cell group has increased relative to the 5 x 10^4 cell culture group, thus at 10, 15 and 25 percent FCS per culture, there was an increase in the inhibition of isotope uptake of 35, 12 and 14 percent respectively, which was not a significant change (P > .05), except for the 25% FCS group which was significant (0.05 > P > .01) and this was due to the small standard error of these values.

Experimental procedures:

3.1.6.2: A plasmacytoma cell suspension was prepared and dispensed into microtitre wells at two cell concentrations per well, 5 x 10^4 and 5 x 10^5 as described previously. The cells were cultured for 16 hours at 37 ^{0}C, 5% CO_2, in 3 groups:

1. without FCS
2. with 10% FCS-HI
3. with 10% Active FCS

Each culture received 0.2 uci of 125IUDR, and was replicated 5 times. The uptake of isotope by cultures was assessed as described previously.

Results:

The effect of increasing the cell culture density and culture incubation time, had a marked effect on isotope uptake by the respective cell cultures, (Table 1.6.2). The highest inhibition of isotope uptake by cell cultures, 70.54%, was present in the 5 x 10^5 cell culture group, cultured with Active FCS; while a relatively

Table 1.6.2 : The mean uptake of 125IUDR by plasmacytoma cultures, cultured for 16 hours. The values are expressed as mean counts per minute (cpm) and as a percent inhibition of uptake. ± Standard error values are included.

	Mean cpm ± S.E.	
Culture group	5×10^4	5×10^5
1	21124 ± 859	22970 ± 1959
2	22863 ± 1441	10599 ± 909
3	22738 ± 451	6766 ± 595
	Percent inhibition ± S.E.	
2	+ 8.23 ± 7.5	53.85 ± 4.57
3	+ 7.64 ± 2.34	70.54 ± 3

+ = stimulation of isotope uptake (no inhibition)

1 = without FCS
2 = with 10% FCS.HI.
3 = with active 10% FCS

lower inhibition of isotope uptake, 53.85%, was present in cultures cultured with FCS-HI. The uptake of isotope in both cultures, (FCS: Active and HI), was significantly different ($P < .01$), from the uptake of isotope by control cultures. In the 5×10^4 cell culture group the uptake of isotope was slightly stimulated but was not significantly different ($P > .05$), from the uptake of isotope by control cultures (0% FCS). The uptake of isotope by control cultures, cultured at both culture densities, was similar ($P > .05$). The cell viability of the high density (5×10^5) culture group, cultured with Active FCS, was not significantly different ($P > .05$), from the cell viability of cultures cultured without FCS.

As described above, (section 3.1.6) the interaction of FCS and BSA with cultures of plasmacytoma and thymocytes at two different cell culture concentrations was assessed. The inhibition of isotope uptake by FCS on plasmacytoma was lower than that produced by BSA, but it was evident that while the inhibitory effects of FCS (at most FCS concentrations) were a function of the cell culture concentration (approximately twice the inhibition at the 1×10^5 cell dose relative to the 5×10^4 cell dose) the inhibitory effects of BSA were not. Similarly the interaction of the thymocytes with FCS at both cell culture doses, were generally alike. Plasmacytoma may be secreting factors that may interact with FCS to produce such an interaction. The cell culture density (3.1.6.2) was increased 10 fold from 5×10^4 to 5×10^5 cells per culture and the cultures were incubated for an extended period with and without FCS. The experiment has shown that the inhibition of isotope uptake was partly due to the increased incubation time and partly to the high cell density in the presence of FCS relative to the low cell density cultures. Thus a putative factor or factors due to

plasmacytoma may be (responsible) interacting with FCS to produce this inhibitory effect. This inhibitory effect may also be due to some factors present in serum.

3.1.7 The relationship between the uptake of 125IUDR by plasmacytoma cultures and the timing of 125IUDR application to cell cultures for different periods of incubation.

Experimental procedures:

A plasmacytoma cell suspension was prepared as described previously; microtitre wells received 1 x 10^5 plasmacytoma cells in 10% FCS and 0.2 uci 125IUDR per well. The cultures were incubated as described earlier. The cultures received 125IUDR at the following timing periods:

(a) 0/4 = cultures received 125IUDR at zero time (at the time of addition of cell suspension to microtitre wells), and were incubated for 4 hours.

(b) 2/4 = cell cultures were initially incubated for 2 hours without 125IUDR; after the 2 hours incubation, 125IUDR was dispensed to cell cultures, and the cell cultures were incubated for a 4 hour period.

(c) 4/4 = cell cultures were incubated for 4 hours without 125IUDR. Following the incubation, 125IUDR was dispensed to cell cultures and the cultures were incubated for a further 4 hour period. The uptake of isotope by cultures was assessed as described earlier.

Results:

The relative uptake of 125IUDR by cells cultured with or without 10% FCS, has increased, as the timing of 125IUDR application to cultures was changed from 0 hour to 2 and 4 hours, (Figure/Table 1.7). The increase in mean cpm for cells cultured without FCS between the 0/4 and 4/4 groups was 2271 counts per minute. The increase in mean cpm for cells cultured with 10% FCS between 0/4 and 4/4 groups was 3382 counts per minute. The relative mean percent inhibition of 125IUDR uptake decreased by around 14%, as the timing of 125IUDR application was changed from 0 hour to 2 and 4 hours, from 47.78% at 0/4 to 33.99% at 4/4 incubation sequence. The respective mean cpm of cells cultured with FCS, was significantly different from the mean cpm of their counterpart cultures, cultured without FCS ($P<0.01$). The inhibition of isotope uptake by FCS was maintained, irrespective of the timing of isotope-culture incubation sequence.

The experiment described above was performed to assess the interactive process between FCS and plasmacytoma at three incubation sequences (0/4, 2/4 and 4/4). The purpose of the experiment was to increase the incubation period before the application of 125IUDR, to note whether this relatively extended incubation period relative to controls (0/4), would produce more inhibition on isotope uptake; as other experiments described later in other sections, have shown that the preincubation of cultures with some batches of FCS, have produced more inhibition on the uptake of isotope.

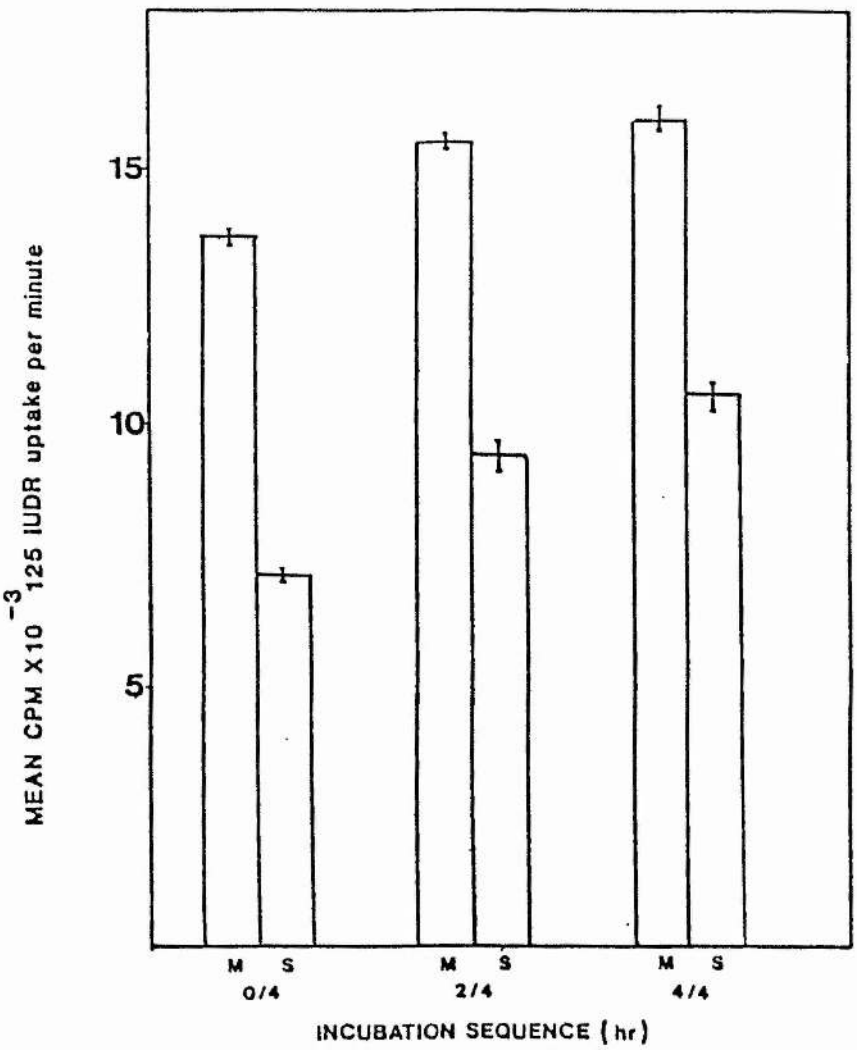


FIGURE 1.7 : The relationship between the uptake of 125IUDR by plasmacytoma cultures, cultured with FCS-HI and the timing of 125IUDR application to cell cultures, at different periods of incubation time .

M = 0% FCS . S = 10% FCS

Table 1.7 : The effects of FCS on the uptake of 125IUDR relative to the timing of 125IUDR introduction into cell cultures, for different periods of incubation. The mean uptake of 125IUDR is expressed as mean counts per minute and as percent inhibition. ± standard error values are included.

	Incubation Period/Sequence		
FCS Concentration	0/4	2/4	4/4
0% 10%	13663 ± 121 7134 ± 73	15485 ± 161 9379 ± 288	15935 ± 226 10517 ± 284
		Percent Inhibition ± S.E.	
	47.78% ± 0.53	39.43% ± 1.86	33.92 ± 1.76

3.1.8: The effects of various types of sera on the uptake of 125IUDR by plasmacytoma cultures.

Experimental procedures:

3.1.8.1: A plasmacytoma suspension was prepared and dispensed into microtitre wells, at a dose of 1 x 10^5 cells/well. Cells were cultured with either (FCS, Human, or Horse) serum, at 10% concentration. Each culture received 0.2 uci of 125IUDR, and was replicated 5 times. The cultures were incubated for 4, 6 and 8 hours, at 37 °C, 5% CO_2. All sera were heat inactivated at 56 °C, 30 minutes , before their use as culture supplements. The uptake of isotope by cultures was assessed as described earlier.

Results:

The mean cpm of cultures, cultured with FCS for 4 hours, was not significantly different (P >.01), from the mean cpm of control cultures, (Table 1.8.1). However, the isotope uptake by cultures, which were cultured with FCS for 6 hours, was inhibited by 38.09%, and was significantly different (P <.01), from the uptake of isotope by control cultures. At 8 hours of incubation, the inhibitory effects of FCS on the uptake of isotope by cultures, were reduced to 13.51%, thus the mean cpm of the respective culture was not significantly different (P > .05), from the mean cpm of control cultures. Horse serum was not inhibitory on the uptake of isotope by cultures, cultured with the respective serum for 4 hours. The mean cpm of the respective cultures at 4 hours incubation, was not significantly different (P >.05), from the mean cpm of control cultures; but at 6 hours of incubation, horse

Table 1.8.1: The mean uptake of 125IUDR (cpm ± S.E.) by plasmacytoma cells cultured for 4, 6 and 8 hours, in FCS, human serum and horse serum. The uptake of isotope is also expressed as percent inhibition.

	Mean cpm ± S.E. and incubation period (hours)		
Serum Type	4	6	8
FCS	9357 ± 146	9729 ± 739	15719 ± 1131
Horse	9113 ± 268	1452 ± 272	3026 ± 162
Human	5600 ± 263	8833 ± 319	12739 ± 160
Controls	9155 ± 498	15716 ± 1065	18175 ± 382
	Percent inhibition ± S.E.		
FCS	+ 2.20 ± 1.6	38.09 ± 4.72	13.51 ± 6.24
Horse	0.46 ± 2.92	90.75 ± 1.74	83.35 ± 1
Human	38.88 ± 2.87	43.79 ± 2	29.90 ± 0

+ = stimulation of uptake (no inhibition)

serum produced a marked inhibition of isotope uptake, which was a reduction of 90.75% in the uptake of isotope by the respective cultures, relative to the uptake of isotope by control cultures, which was a significant difference (P <.01). The effective inhibition by horse serum on the uptake of isotope by cultures was maintained at 8 hours of incubation; the mean cpm of the respective cultures, was significantly different (P <.01), from the mean cpm of control cultures. Human serum, at 4 hours, produced 38.8% inhibition of isotope uptake by cultures, which was significantly different (P < .01), from the uptake of isotope by control cultures. There was a relative change in the inhibitory effects of human serum on isotope uptake by cell cultures, at 6 and 8 hours of incubation; the mean cpm of the respective cultures was significantly different (P <.01), from the mean cpm of control cultures. The cell viabilities of cultures, cultured with horse or human serum (8 hour culture) were not significantly different from the viabilities of cells cultured without serum (P > .05). The inhibitory effects of sera, as described above, are a function of incubation time;, as with this batch of FCS used in this experiment, the inhibitory effects of FCS on isotope uptake by cell cultures were evident at 6 hours instead of 4 hours, but with the usual pattern of a decrease in inhibition with further incubation. Horse serum, as FCS, was not inhibitory at 4 hours, but was so at 6 hours, with an inhibition which was approximately twice that produced by the other two sera, which were nearly similar in their inhibitory effects. At 8 hours of incubation, the inhibitory effects of horse serum were about three fold more than human serum, and about six fold more than FCS.

Experimental procedures:

3.1.8.2: A cell suspension of plasmacytoma was prepared and dispensed into microtitre wells at 1 x 10^5 cells per culture. Cells were cultured in the following [heat-inactivated 56 °C, 30 minutes, and Active] sera: FCS, CD1 and CBA mice sera, rat serum, BSA (bovine serum albumin) and HSA (human serum albumin) at a concentration of 10% and 20% per culture; together with control cultures, cultured without serum . All cell cultures received 0.2 uci 125IUDR per culture, and were replicated 5 times. Cultures were incubated for a period of 4 hours as described previously. The final volume per culture was 200 ul. The uptake of isotope was assessed as described earlier.

Results:

The uptake of 125IUDR by plasmacytoma cultures in the presence of various types of sera has been described, (Tables 1.8.2.A and 1.8.2.B). In the 10% [heat-inactivated sera] group the mean cpm range for the FCS, rat and mice sera was between 11087 to 14165, the respective percent inhibition of 125IUDR uptake ranged from 40.11% to 53.12%; all were significantly different (P <.01), from the mean cpm of cells cultured without serum.

The inhibitory effects of rat and CD1 sera were not significantly different from FCS, while the inhibitory effect of CBA serum was different(P <.01). The mean cpm of cultures in the 20% heat inactivated sera ranged between 7328 and 9535; the respective percent inhibition of 125IUDR uptake ranged between 59.68% and 69.01%. The mean cpm of cultures, cultured with rat, CBA or CD1 sera were not significantly different (P >.01) , from from the mean cpm of cultures, cultured with FCS.

Table 18.2.A: The mean uptake of 125IUDR expressed as counts per minute with standard error (cpm ± S.E.) by plasmacytoma cells cultured for 4 hours, in different types of sera at 10% and 20%, and without serum.

	Mean cpm ± S.E. Serum concentration			
	HI		Active	
Serum Code	10%	20%	10%	20%
FCS 1	14165 ± 478	7395 ± 723	8497 ± 278	7262 ± 935
Rat 2	14154 ± 381	9535 ± 1268	15450 ± 915	9150 ± 795
CBA 3	11087 ± 748	9397 ± 516	8887 ± 432	4708 ± 648
CDI 4	12965 ± 2096	7328 ± 442	9370 ± 548	6359 ± 531
BSA 5	-	-	13483 ± 2287	10515 ± 707
HmSA 6	-	-	10973 ± 1115	7122 ± 648
7	Without serum (medium controls) = 23653 ± 1939			

HI = heat inactivated serum
Active = non-heat-inactivated serum

Table 1.8.2.B: The percent inhibition (± S.E.) of 125IUDR uptake by plasmacytoma cells, for data described in Table 1.8.2.A.

Serum Concentration	Percent inhibition ± S.E. Serum Code						
	FCS 1	Rat 2	CBA 3	CDI 4	BSA 5	HmSA 6	Controls 7
10% HI	40.11% ± 2	40.15% ± 1.6	53.12% ± 3.2	45.18% ± 4	-	-	0
20% HI	68.73% ± 3	59.68% ± 5.4	60.26% ± 2.2	69.01% ± 1.8	-	-	
Active 10%	64.4% ± 1.2	34.67% ± 3.9	62.42% ± 2	60.38% ± 2.3	42.99% ± 4.3	53.6% ± 4.7	
20%	69.29% ± 4	61.31% ± 3.4	80.09% ± 2.8	73.11% ± 2.2	55.54% ± 3	69.88% ± 2.7	

In the 10% [Active sera] group, the mean cpm of cultures, cultured with FCS, rat, mice, BSA and HSA ranged from 8497 to 15450; the respective percent inhibition of 125IUDR uptake ranged from 34.67% to 64.4%, all were significantly different (P <.01), from the mean cpm of cells cultured without serum.

The mean cpm of cultures, cultured with rat or BSA sera were significantly different (P <.01), from the mean cpm of cultures, cultured with the FCS, while the respective mean cpm of cultures, cultured with CBA, CD1 and HSA was not different (P >.01), from the mean cpm of cultures, cultured with FCS.

The mean cpm in the 20% [Active sera] group, ranged between 4708 and 10515, the respective percent inhibition of 125IUDR uptake ranged from 55.54% to 80.09%. The respective mean cpm of cultures in all types of sera was not significantly different (P > .01), from the mean cpm of cultures, cultured with FCS.

The cell viabilities of cultures, cultured in the respective 20% [Active serum] type, were not significantly different (P >.05) from their control counterparts cultured without serum.

The experiments described above, (sections 3.1.8.1 and 3.1.8.2) were assessing the effects of sera other than FCS, on the uptake of isotope by cell cultures, and compare and contrast these effects with FCS. A kinetic study, (section 3.1.8.1, effects of serum and period of incubation) was performed to assess the pattern of isotope uptake in the presence of FCS, human and horse sera. Human serum was generally similar to FCS in the pattern of isotope uptake inhibition, as in both sera the inhibitory effects were decreasing as the period of incubation was increasing. Horse serum was different from FCS, as the inhibition of isotope uptake was

relatively constant at extended periods of incubation.

The effects of rat serum, mice sera, BSA and HSA on isotope uptake were also assessed in ralation to the effects of FCS, (section 3.1.8.2). Both sets of experiments have shown that other sera were also inhibitory, thus the inhibition of isotope uptake is not a characteristic effect of FCS.

3.1.9: The relationship between the uptake of 125IUDR by plasmacytoma cultures, and the isotope activity remaining in microtitre wells used for the plasmacytoma cultures.

Experimental procedures:

A suspension of plasmacytoma cells was prepared and dispensed into microtitre wells at a cell culture dose of 1 x 10 cells/well. Cells were cultured with 0%, 5%, 10%, 15% and 25% FCS-HI [batch A], as described in section 1.10.B. Each culture received 0.2 uci 125IUDR, and was replicated 5 times. Cultures were incubated for 4 hours, at 37 ^{0}C, 5% CO_2, as described previously. The cultures were harvested as described in materials and methods. The culture plates were dried, and individual wells were sawn off and were examined for their radioactivity content in a gamma spectrometer.

Results:

The results of the isotope uptake curves, (Figure/Table 1.9) are as follows: Curve A , (column A, Table 1.9) represents the actual inhibition produced by FCS on the uptake of isotope by cell cultures. Curve B, represents the difference (expressed in mean

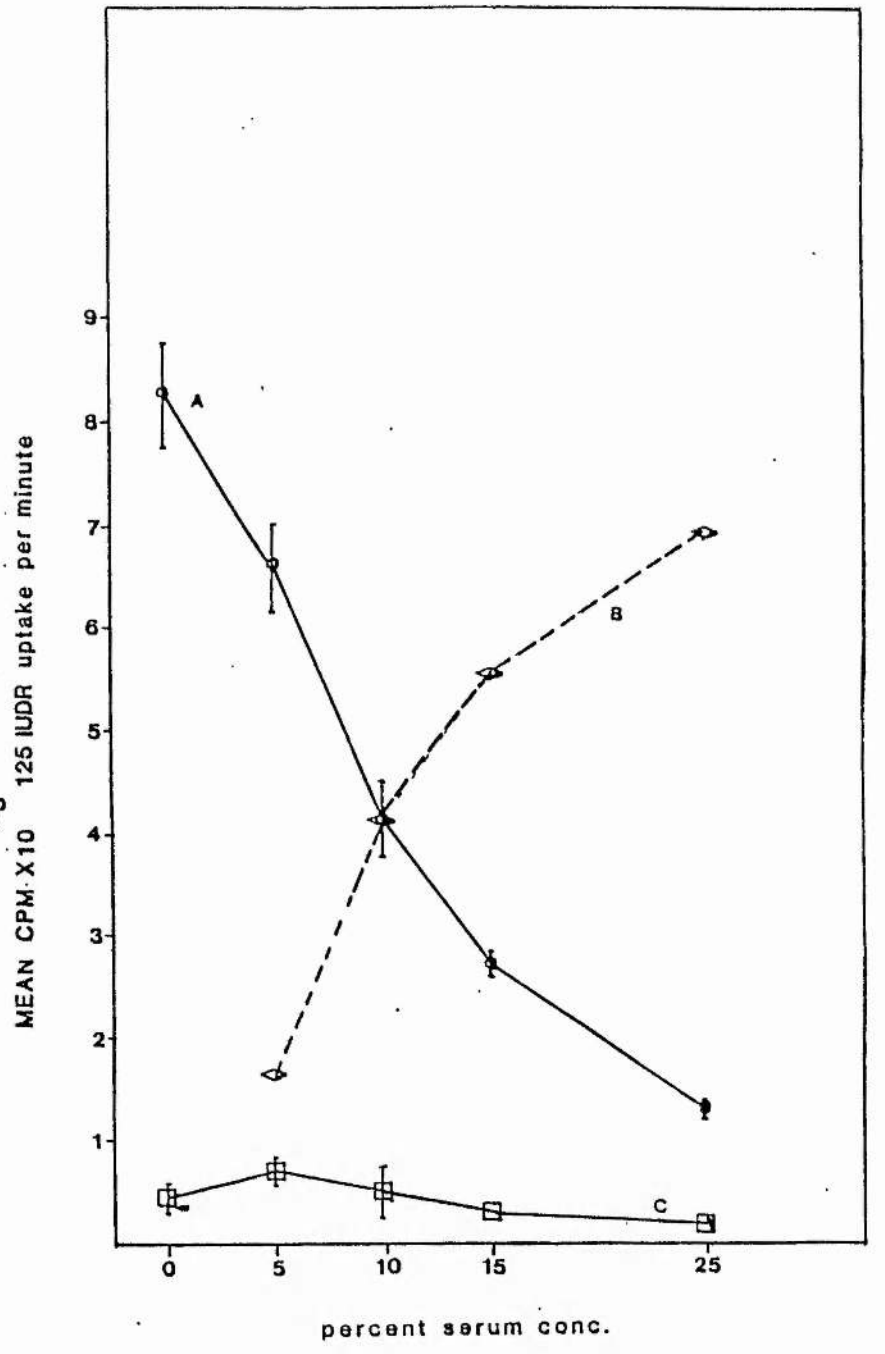


FIGURE 1.9 : The uptake of 125IUDR by plasmacytoma cultures, cultured with FCS-HI for 4 hours.
A: actual amount of isotope uptake by cultures
B: expected amount of isotope uptake inhibition due to serum
C: actual amount of radioactivity remaining in microtitre wells

Table 1.9 .: The effect of FCS concentration on the uptake of 125 IUDR by plasmacytoma cells, and the amount of radioactivity remaining in microtitre culture wells. Cell cultures were incubated for 4 hours. The values quoted in the table are the uptake of 125IUDR expressed as mean counts per minute (cpm) and as percent inhibition, the standard error values are included.

FCS concentration	(A) Curve 1 mean cpm ± S.E.	Percent inhibition	(B) Curve 2 cpm	(C) Curve 3 cpm	(D) Percent of total:
0%	8279 ± 227	0.0	0.0	688 ± 165	0.0
5%	6623 ± 430	19.99 ± 5.2	1632	711 ± 142	43.57
10%	4149 ± 418	49.88 ± 5	4297	520 ± 226	12.11
15%	2729 ± 111	67.03 ± 1.3	5930	307 ± 35	5.18
25%	1311 ± 100	84.16 ± 1.2	7454	202 ± 43	2.71

A: The actual amount of isotope uptake by cell cultures.
B: The expected amount of isotope uptake, inhibition due to FCS.
C: The actual amount of radioactivity remaining in the microtitre wells.
D: Values in (C) expressed as a percentage of values in (B)

* See text for details.

cpm) between the uptake of isotope by the respective cultures and cultures cultured without FCS. Curve C, represents the amount of radioctivity remaining in culture wells, after cell harvest. The values in column D, are the percentages of values in column C, in terms of values in column B . As the FCS concentration per culture has increased, the uptake of isotope by plasmacytoma cultures decreased, as in Curve 1. The highest mean cpm was for cultures, cultured without FCS; the lowest mean cpm was for cultures, cultured with 25% FCS. The inhibition by FCS, on isotope uptake by plasmacytoma cultures, ranged from 19.99% at 5% FCS per culture, to 84.16% at 25% FCS per culture. An analysis of variance and Dunnett's test (2 tail, P = .01), between the mean cpm of cultures, cultured with 0%, 5%, 10%, 15% and 25% FCS, was significant, (Anovar. F ratio = 101.17, P <.01). The mean cpm of cultures, cultured without FCS were significantly different from mean cpm of cells cultured with 25%, 15%, 10% and 5% FCS . The mean cpm of cells cultured with 5% FCS was significantly different from the mean cpm of cultures, cultured with 25%, 15% and 10% FCS. The mean cpm of cells cultured with 10% FCS was significantly different from the mean cpm of cultures cultured with 25% and 15% FCS. The mean cpm of cells cultured with 25% was also different from the mean cpm of cells cultured with 15% FCS. The quantity of isotope uptake inhibition, expressed in mean cpm in Curve 2, has increased as the FCS concentration per culture increased, from 1632 mean cpm at 5% FCS to 7454 mean cpm at 25% FCS . The mean cpm available in culture wells which were used for plasmacytoma cultures has decreased as the concentration of FCS per culture increased. Thus the mean cpm for the 0% and 5% FCS culture wells were 688 and 711 respectively, which has decreased to 202 at 25% FCS; although an Anovar. between the mean cpm of culture wells was

not significant, (F.ratio = 2.57, P > .05). When the mean cpm of culture wells was expressed as a percentage of the total mean cpm inhibition (Curve 3 vs Curve 2) one notes, that the reduction in isotope uptake by cultures, accounted for, in part, by isotope activity remaining in the wells, was highest at 5% FCS (43.57% inhibition) and lowest at 25% FCS (2.71% inhibition).

The experiment described above (section 3.1.9), was performed to assess the effects of serum concentration on the amount of isotope activity remaining in the microtitre culture dishes. The results have demonstrated that when the concentration of serum increases in the culture environment, the amount of isotope activity remaining in culture dishes decreases. Therefore the decrease in isotope activity recovered in harvested cultures (i.e. the amount of isotope uptake inhibition), was not due to a decrease (in part) in isotope availability to cell cultures, because of isotope remaining attached to culture dishes.

3.2: THE EFFECTS OF UIF ON CELLULAR PROLIFERATION IN INVITRO CELL CULTURES

3.2.1.1: The effects of RAT UIF on DNA synthesis as assessed by the inhibition of 125 IUDR uptake by cell cultures.

Experimental procedures:

Rat Thymus UIF, Rat Spleen UIF were prepared from cells of Rat thymus and spleen, (50×10^6 thymocytes or spleen cells were incubated in 5 ml of RPMI without FCS in 50 mm diameters tissue culture sterilin petri dishes for 16 hours at 37°C); several batches of thymus UIF and spleen UIF were prepared. A cell suspension of plasmacytoma was prepared and dispensed into microtitre wells at 1×10^5 cells per well in 10% FCS-HI. Wells received 5, 10, 20, and 40 ul per well of Thymus UIF (TC-UIF), other sets of wells received RCW.UIF spleen UIF (RWC = whole spleen, Red cells and white cells UIF), and WCF.UIF spleen UIF (WCF = white cell fraction without red cells). Each well received 0.2 uci of 125 IUDR; each UIF concentration was replicated in 5 wells and cell cultures were incubated for a 4 hour period.

The final volume per well was 200 ul. The effect of each type of UIF on cell proliferation, was tested individually to assess the inhibition, as measured by the uptake of 125 IUDR. All cultures with UIF have control cultures, in which cells were cultured without UIF. The UIFS described in Section 2.1.1., and in Figure 2.1.A are coded as UIF batch 1.

Results:

The general trend of 125 IUDR uptake inhibition was linear, which increased as the amount of UIF per culture well increased, as described in Figure/Table 2.1.A . The Thymus UIF showed small inhibitions at 5 and 10 ul per well, of 0.3% and 6.94% respectively. The inhibition increased to 15.65% and 25.08% at 20 and 40 ul per

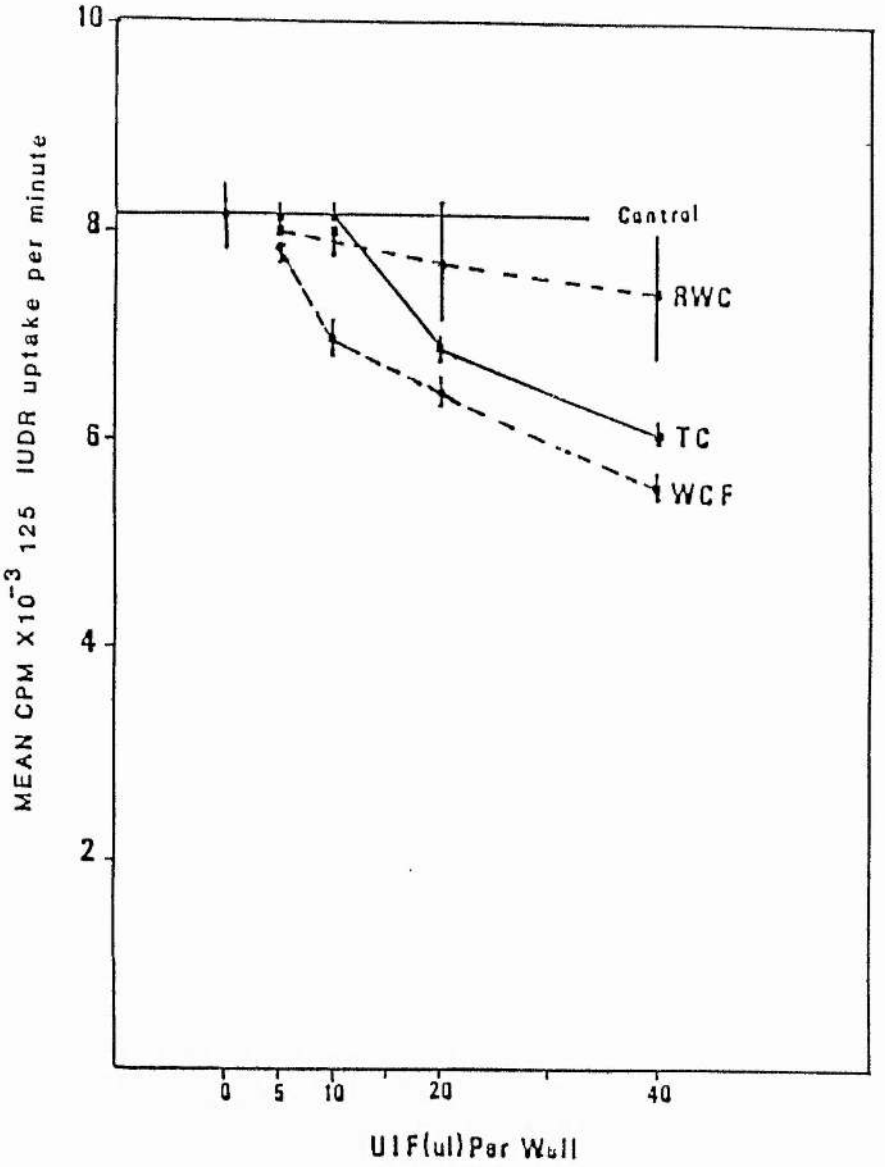


FIGURE 2.1A: The uptake of 125IUDR by plasmacytoma cultures, cultured with FCS -HI and different concentrations of UIF for 4 hours.

Rat UIF: RWC (spleen; red and white cells) UIF
WCF (spleen; white cell fraction) UIF
TC (thymus cells) UIF

Table 2.1.A: The effect of Rat UIf on the uptake of 125IUDR by plasmacytoma cells cultured for four hours. The uptake of 125IUDR is expressed as mean counts per minute (cpm) and as percent inhibition with ± S.E. values included.

	Thymus UIF.TC	Spleen UIF.RWC	Spleen UIF.Wcf.
UIF Concentration ul per well	cpm ± S.E.	cpm ± S.E.	cpm ± S.E.
0	8128 ± 121	8128 ± 121	8128 ± 121
5	8104 ± 41	8063 ± 204	7866 ± 144
10	7564 ± 225	8024 ± 268	6955 ± 169
20	6856 ± 149	7669 ± 583	6463 ± 122
40.	6090 ± 89	7394 ± 564	5566 ± 103
	Percent Inhibition ± S.E.	Percent Inhibition ± S.E.	Percent Inhibition ± S.E.
0	0.0	0.0	0.0
5	0.3 ± 0.5	0.8 ± 2.52	3.21 ± 1.77
10	6.94 ± 2.81	1.27 ± 3.3	14.42 ± 2
20	15.65 ± 1.83	5.64 ± 7.2	20.47 ± 1.5
40	25.08 ± 1.1	9.02 ± 6.96	31.51 ± 1.3

UIF.TC = Thymocytes UIF; UIF.RWC = Spleen red and white cells UIF; UIF.Wcf = spleen white cell fraction UIF without red cells.

well. At 5 and 10 ul UIF per well the inhibition of 0.30% and 6.94% were not significantly different from control values of cells cultured without UIF, (P>.05, t=0.0695 and .0019 respectively). At the 20 ul UIF per well the inhibition of 125 IUDR uptake was significant, (0.05>P>.01 t=3.246,). At 40 ul per well, the inhibition of isotope uptake was significant (P>.01, t=5.437).

The spleen (UIF.RWC) fraction showed small inhibitions throughtout the whole range of UIF concentrations per well. The inhibitions ranged from 0.8% at 5 ul to 9.02% at 40 ul, and at all UIF concentration the inhibition was not significant, (P>.05, t=0.154, 0.226, 0.664 and 1.087 respectively from 5 ul UIF to 40 ul UIF).

The spleen (UIF.WCE) showed inhibitions which were relatively higher than the other 2 UIFS as in table 2.1A. The inhibitions increased with increasing UIF concentration per well from 5 ul to 40 ul.

The inhibitions at 40 ul and 20 ul UIF were significant (t-2tail P=<.01, 8df, 4.28 and 7.01 respectively), at 10 ul UIF the inhibition was significant (0.05>P>.01, t=2.88), while at 5 ul UIF the inhibition was not significant (P>.05, t=0.659). The effects of UIF on cell viability are described in table 2.2C.

The percent inhibition has been determined as described in section 3.1

3.2.1.2: The effects of RAT UIF on DNA synthesis and the inhibition of 125 IUDR uptake by cell cultures.

Experimental procedures:

Rat UIF was prepared from cells of the thymus or spleen (5×10^7 cells in 5 ml RPMI without FCS dispensed into sterile 50 mm tissue culture sterlin petri dishes, and were incubated for 16 hours at 37°C) as has been described earlier. Several batches of TC-UIF, WRC-UIF and WCF-UIF were prepared and tested for their inhibitory effects. Three batches of each type of UIF have been used for the experiment described in this section 2.1.2. The batches are coded as: 18.2, 19.2 and 29.2. Cell suspensions of plasmacytoma and of mouse thymocytes were dispensed into their respective microtitre wells, each at a concentration of 1×10^5 cells per well. Cell cultures were divided into two parts, those cultured without FCS, and those cultured with 10% FCS-HI. Three batches of each type of UIF were assessed. Wells received 20, or 40 ul of each batch of UIF and 0.2 uci of 125 IUDR; each UIF concentration was replicated 5 times. Cell cultures were incubated for 4 hours. The final volume per well was 200 ul. All cultures have control cultures, in which cells were cultured without UIF.

Results:

The difference in isotope uptake, expressed as cpm (mean counts per minute) between plasmacytoma cultures cultured without FCS (medium controls, 6225 cpm), and cells cultured with FCS 10% (FCS controls, 3765 cpm) was significant, t=4.76, 2tail, P<.01 with 10% FCS causing a 39.51% inhibition of 125 IUDR uptake as described in Table 2.1.B1 . The batch total mean cpm per UIF group for cells cultured without FCS were similar at 5869 to 5889 cpm, for UIF.TC at 20 and 40 ul

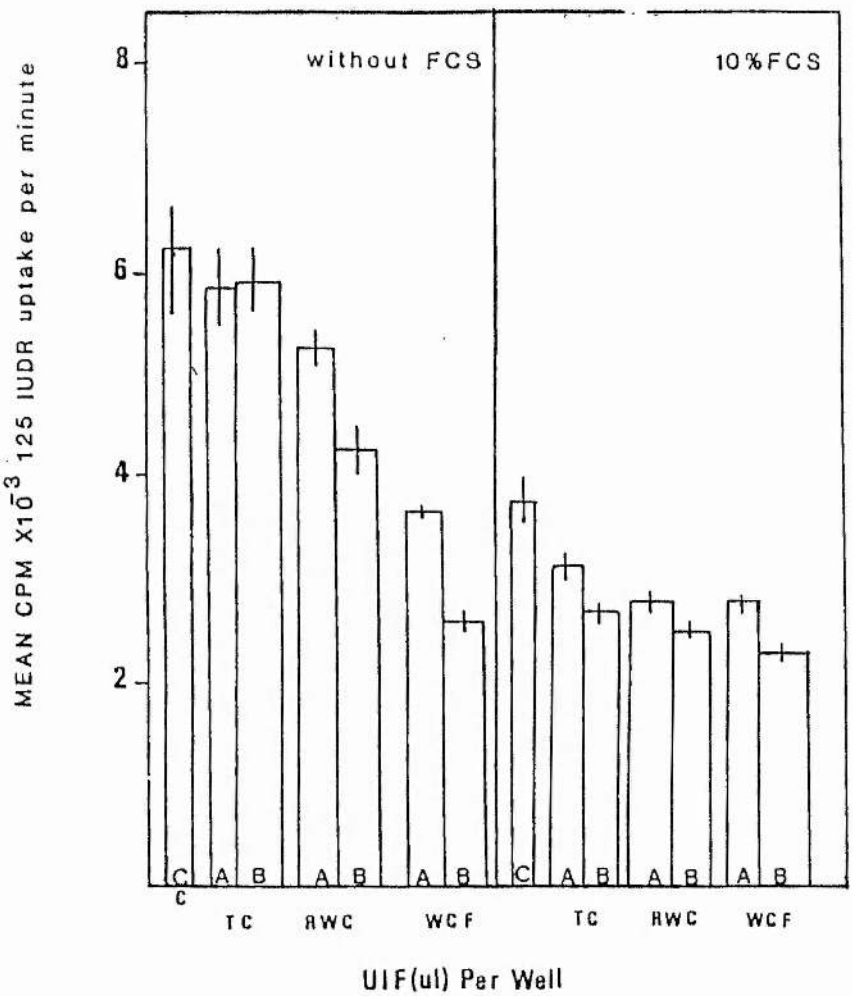


FIGURE 2.1.B1: The uptake of 125IUDR by plasmacytoma cultures, cultured without and with FCS-HI, for 4 hours, with rat UIF.
RWC: spleen red and white cells UIF
WCF: spleen white cell fraction UIF
TC : thymus cells UIF
A: 20ul, B: 40ul .

Table 2.1.B1: The effects of Rat UIFS: TC, RWC and Wcf on the uptake of 125 IUDR by plasmacytoma cultures, cultured for 4 hours, without and with 10% FCS. The values are expressed as mean cpm, with ± S.E. values included for each UIF batch. The total mean cpm per UIF group of 3 batches is included. The respective mean percent inhibitions, with ± S.E. values are included.

		Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	UIF batch	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
20 ul	18.2	6202 ± 237	0.3	3149 ± 102	16.3
TC	19.2	5218 ± 367	16.1	3100 ± 238	17.6
	29.2	6187 ± 301	0.69	3127 ± 103	16.9
Batch mean		5869 ± 325	5.66 ± 5.22	3125 ± 14	16.93 ± 0.57
40 ul	18.2	6195 ± 57	0.4	2847 ± 251	24.3
TC	19.2	5271 ± 228	15.3	2635 ± 126	30
	29.2	6201 ± 264	0.3	2664 ± 140	29.2
Batch mean		5889 ± 309	5.33 ± 4.98	2715 ± 66	27.83 ± 1.78
20 ul	18.2	5114 ± 153	17.8	2926 ± 169	22.2
RWC	19.2	5548 ± 175	10.8	2810 ± 227	25.3
	29.2	5072 ± 206	18.5	2722 ± 172	27.7
Batch mean		5244 ± 152	15.7 ± 2.46	2819 ± 59	25.06 ± 1.59
40 ul	18.2	3900 ± 32	37.3	2342 ± 130	37.7
RWC	19.2	4426 ± 168	28.8	2509 ± 120	33.35
	29.2	4399 ± 207	29.3	2622 ± 72	30.3
Batch mean		4241 ± 171	31.8 ± 2.75	2491 ± 81	33.78 ± 2.14

Table 2.1.B1 (continued)

		Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	UIF batch	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
20 ul	18.2	3722 ± 43	40.2	2921 ± 137	22
Wcf	19.2	3737 ± 110	39.9	2706 ± 232	28.1
	29.2	3595 ± 122	42.2	2775 ± 131	26.2
Batch mean		3684 ± 45	40.76 ± 0.72	2800 ± 63	25.5 ± 1.79
40 ul	18.2	2686 ± 88	56.8	2246 ± 177	40.3
Wcf	19.2	2352 ± 74	62.2	2477 ± 92	34.2
	29.2	2707 ± 115	56.5	2166 ± 56	42.4
Batch mean		2581 ± 115	58.5 ± 1.85	2296 ± 93	38.96 ± 2.46
Medium Control without FCS		6225 ± 493	0.00	10% FCS Control 3765 ± 180	0.00

UIF.TC = Thymocytes UIF; UIF. RWC = spleen; red and white cells UIF; UIF.Wcf = spleen : white cell fraction UIF without red cells.

respectively, with corresponding inhibitions of around 5% for both; which were not significantly different from medium controls (t=0.872 and 1.028 respectively, P>.05).

For RWC.UIF at 20 ul, the respective batch total mean percent inhibition of isotope uptake was 15.7%, which increased twofold to 31.8% when the UIF concentration was increased to 40 ul. Both inhibitions were significantly different from medium controls, (t=3.027 and 6.57 respectively, P<.01).

For the WCf.UIF there was a 40.7% batch total mean percent inhibition at 20 ul, which increased moderately to about 58% when the UIF concentration was doubled to 40 ul per well. Both values were significantly different from medium controls (t=8.94 and 12.81 respectively, P<.01). The highest inhibitions were by the WCf.UIF group, followed by RWC.UIF and the least inhibitory was the TC.UIF .

For cells cultured in 10% FCS, UIF.TC produced around 17% inhibition of isotope uptake at 20 ul per well, which increased to about 28% when the UIF concentration per well was doubled. Both inhibitions were signficantly different from 10% FCS culture controls, (t=3.56 and 5.22 respectively, P<.01).

For UIF.RWC at 20 ul per well, the inhibition was 25% which increased to 33.7% when the UIF concentration per well was increased to 40 ul.

Both were significantly different from 10% FCS controls (t=4.37 8.67 respectively P<.01).

For UIF.WCf the amount of inhibition produced at 20 or 40 ul per well were similar to RWC.UIF and were in the region of 25 to 38.9% repectively.

Both were signficantly different from 10% FCS controls (t=4.92

and 9.95 respectively P<.01). At 20 ul per well, an analysis of variance (Anovar) between the respective mean percent inhibitions for the 3 UIFS in 10% FCS showed significant differences (F=12.19, P<.01).

The WCf.UIF was significantly different from TC.UIF, but not different from RWC.UIF while the RWC.UIF was significantly different from TC.UIF . The significance of the difference between the UIF types was assessed by using the multiple range test - short significance ranges (MRT-SSR).

For the 40 ul per well, the values of percent inhibitions for the 3 UIFS showed no signficant differences in an Anovar at (P=<.01, F=6.26 but were significant at P=<.05). The MRT-SSR showed no differences between the means.

For cells cultured without FCS, the differences between the 3 UIFS (TC, RWC, WCf) on 125 IUDR uptake inhibitions at 20 ul per well were significantly different among their values in an Anovar at (P=<.01, F=29.11). WCf.UIF was significantly different from RWC and TC.UIFS, while RWC was not different from TC.UIF using the MRT-SSR .

At the 40 ul per well the 3 UIFS were significantly different from each other, using an Anovar, (P<.01, F=59.15) and the MRT-SSR.

A comparison between the percent inhibitions caused by the three UIFS administered to cells cultured with or without FCS using a 2tail t-test comparison with 4 df, was as follows:

The TC.UIF at 20 and 40 ul cultured without FCS or in 10% FCS were not significantly different at P<.01, although the 40 ul was more inhibitory in 10% FCS than its counterpart without FCS, but was significant at (0.05>P>.01)

The percent inhibition produced by RWC.UIF at 20 or 40 ul, without or with 10% FCS were not significantly different from each other (P<.01), although RWC 20 ul in FCS was significantly greater than its counterpart without FCS, (0.05>P>0.01).

The percent inhibition produced by the WCf.UIF at 20 ul and 40 ul, without FCS, or with 10% FCS; the inhibitions were significantly greater in cells cultured without FCS, compared to cells with 10% FCS (P<.01).

The inhibitions of isotope uptake by UIF for cells cultured without FCS show that WCf.UIF produced the highest inhibition compared to RWC and TC.UIFS, however this was not an absolute case for cells cultured with 10% FCS. The calculation of percent uptake inhibition has been determined as described in Section 3.1. The percent inhibitions for cell cultures without FCS were determined in terms of control cultures without FCS, while for those cultured with 10% FCS, the percent inhibitions were determined in terms of control cultures (10% FCS).

The difference in isotope uptake expressed as cpm between mouse thymocyte cultures, cultured without FCS (medium controls, 210) and cultured with FCS (10% FCS controls 240) as described in Table 2.1.82 was 14.28% which was not significant (t=1.76, P>.05).

For cells cultured without FCS UIF.TC produced similar inhibitions of isotope uptake of approximately 16% at 20 and 40 ul UIF per well. For RWC.UIF there was around 17% inhibition at 20 ul UIF which increased to 21% at 40 ul UIF per well. The highest inhibitions

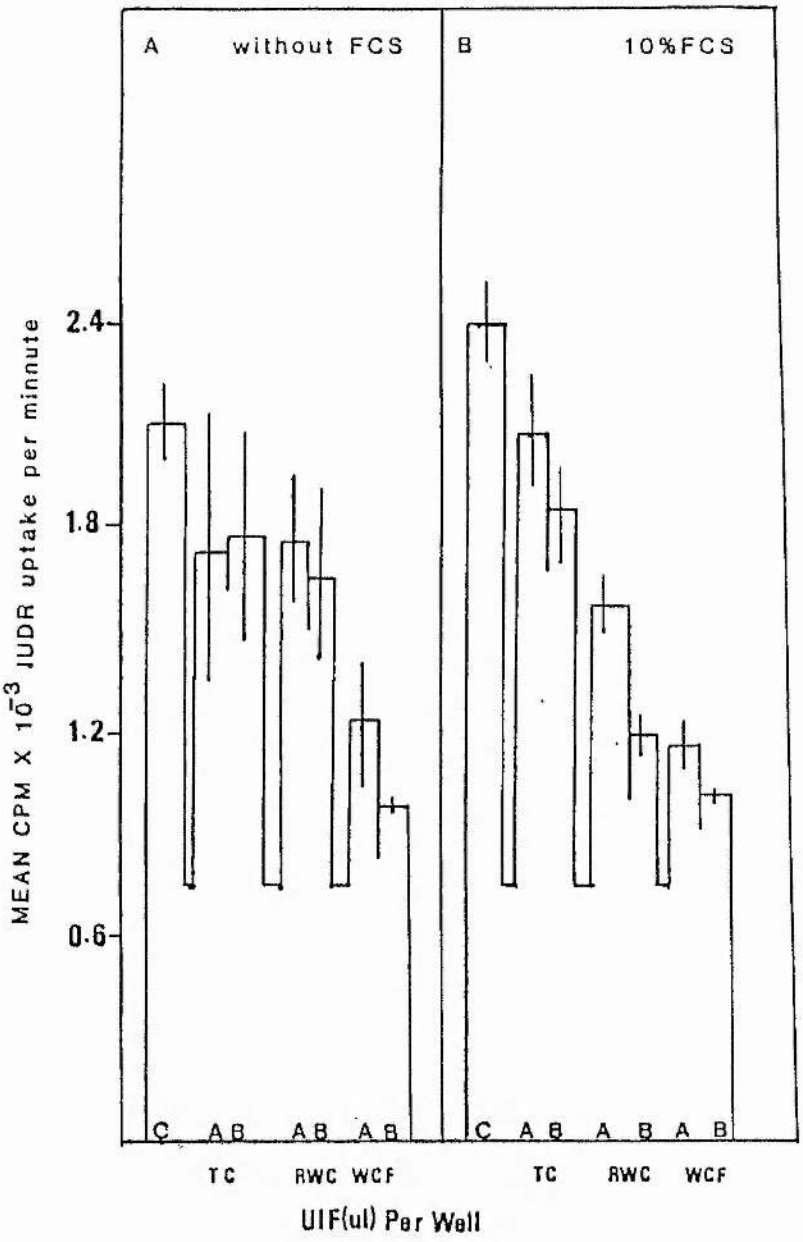


FIGURE 2.1.B2: The uptake of 125IUDR by mouse thymocyte cultures, cultured without and with FCS-HI, for 4 hours, with rat UIF.
RWC: spleen red and white cells UIF
WCF: spleen white cell fraction UIF
TC : thymus cells UIF. A: 20ul, B: 40ul

Table 2.1.B2: The effects of Rat UIF on the uptake of 125IUDR by mouse thymocyte cultures, cultured for 4 hours; without FCS and with 10% FCS. The values are expressed as mean cpm (counts per minutes) with ± S.E. values. The total mean cpm per UIF group of 3 batches, is included. The respective mean percent inhibitions with ± S.E. are included.

		Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	UIF batch	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
20 ul	18.2	207 ± 28	1.42	240 ± 22	0
TC	19.2	100 ± 13	47.61	190 ± 14	20.83
	29.2	210 ± 16	0	187 ± 11	22.08
Total mean		172 ± 36	16.34 ± 15.65	205 ± 17	14.03 ± 7.16
40 ul	18.2	209 ± 5	0.47	206 ± 9	14.16
TC	19.2	114 ± 13	45.71	190 ± 13	20.83
	29.2	208 ± 25	0.95	156 ± 5	35
Total mean		177 ± 31.53	15.71 ± 15.01	184 ± 14	23.33 ± 6.15
20 ul	18.2	142 ± 10	32.38	174 ± 13	27.5
RWC	19.2	210 ± 20	0	146 ± 27	39.16
	29.2	172 ± 8	18.09	153 ± 13	36.25
Total mean		174 ± 19	16.82 ± 9.37	157 ± 8	34.30 ± 3.5
40 ul	18.2	120 ± 10	42.85	106 ± 19	55.83
RWC	19.2	210 ± 8	0	124 ± 13	48.33
	29.2	167 ± 11	20.47	128 ± 26	46.66
Total mean		165 ± 26	21.10 ± 12.38	119 ± 6	50.27 ± 2.82

Table 2.1.B2: (continued)

		Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	UIF batch	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
20 ul	18.2	89 ± 7	57.61	112 ± 9	53.33
Wcf	19.2	148 ± 6	29.52	133 ± 22	44.58
	29.2	137 ± 10	34.76	106 ± 7	55.83
Total mean		124 ± 18	40.63 ± 8.63	117 ± 8	51.24 ± 3.41
40 ul	18.2	103 ± 15	50.95	102 ± 11	57.5
Wcf	19.2	102 ± 4	51.42	106 ± 12	55.83
	29.2	93 ± 11	55.71	100 ± 17	58.33
Total mean	19 ± 3	19 ± 3	52.69 ± 1.51	102 ± 2	57.22 ± 0
Medium Control cpm = 210 ± 12				10% FCS control = 240 ± 12	

UIF.TC = Thymocytes UIF; UIF.RWC = Spleen : red and white cells UIF; UIF.Wcf = Spleen : white cell fraction UIF without red cells.

were produced by WCf.UIF at 20 and 40 ul per well which were 40 to 56% repectively. The mean cpm of TC.UIF at 20 and 40 ul was not different from their culture controls mean cpm (t=1.84 and 2.06 respectively P>.05), while the mean cpm of RWC.UIF at 20 ul was significant at (0.05>P>.01, t=2.44) and at 40 ul significant at (P<.01, t=3.75). The mean cpm of WCf.UIF at 20 and 40 ul was different from the mean cpm of their medium controls (t=8.38 and 9.147 respectively P<.01).

For cells cultured with 10% FCS, TC.UIF produced 14% inhibition at 20 ul per well, which increased to 23% at 40 ul per well. At 20 ul UIF the inhibition was not significant, (t=1.99 P>.05), while for TC 40 ul. The inhibition was significant at (P<.01, t=4.97).

The mean cpm of RWC.UIF at 20 and 40 ul and WCf.UIF 20 and 40 ul were all different from the mean cpm of 10% FCS cultures controls, (t=4.30, 5.81 and 8.50, 9.12 respectively P<.01).

The highest inhibitions were by the WCf.UIF group followed by (RWC.UIF), and the least inhibitory was the TC.UIF .

For cells cultured without FCS, the total mean percent inhibition at 20 ul per UIF, were not different from each other in an Anovar, (P>0.05 F=1.22). Similarly, the total mean percent inhibitions produced by 40 ul of UIFS, TC, RWC and WCf were not different from each other in an Anovar (P<.05, F=0.697).

For cells cultured in 10% FCS, the total mean percent inhibition produced by 20 ul of UIFS, TC, RWC and WCf were significantly different in Anovar; (P<.01, F=13.86) where WCf.UIF was different from TC.UIF but not from RWC.UIF while RWC.UIF was not different from TC.UIF using the Multiple Range Test - Shortest Significant Ranges MRT-SSR.

The total mean percent inhibitions of UIFS TC, RWC and WCE at 40 ul were significantly different in an anovar, ($P<.01$, $F=20.58$), where both WCE and RWC UIFS were different from TC.UIF, while WCE.UIF and RWC.UIF were not different, using the NRT-SSR .

A comparison between the mean percent inhibitions caused by the three UIFS administered to cells cultured with or without FCS using a 2tail t-test has been described, (Table 2.1.2).

There was no significant difference in the mean percent inhibitions of isotope uptake between cells cultured with or without 10% FCS at 20 ul and 40 ul of TC.UIF .

For the RWC.UIF at 20 and 40 ul, the percent inhibitions in cells cultured in 10% FCS were significantly greater than their counterparts cultured without FCS at $P<.05$. For the WCE.UIF at 20 ul and 40 ul, the percent inhibitions for cells cultured with FCS were greater than for cells cultured without FCS at ($P<.05$) for 20 ul and ($P<.01$) for 40 ul UIF.

As described above, (section 3.2.1.2) the inhibitory effects of UIF on cell cultures have been asessed. The results have demonstrated that WCF.UIF produced the highest inhibition of radioisotope uptake at both UIF concentrations (20 and 40 ul) in both culture environments (with and without FCS). The results have demonstrated that the interbatch variation (in radioisotope uptake inhibition) was small within each group of batches, except for the TC.UIF group in which the interbatch variation was relatively large, (i.e. some TC.UIF batches were more inhibitory than others), as assessed on plasmacytoma cultures, cultured without FCS. However, this interbatch variation within the TC.UIF group was minimized when the inhibition of radioisotope uptake was assessed in cultures containing FCS. Thus FCS was potentiating the weak TC.UIF batches 18.2 and 19.2, rendering them as inhibitory as TC.UIF batch 19.2. Similarly, the uptake inhibition by RWC.UIF in the presence of FCS was relatively more than the uptake inhibition available in cultures cultured without FCS. However, the effect of FCS on the WCF.UIF group was different, as the inhibition of radioisotope uptake was relatively reduced in cultures containing FCS, relative to cultures cultured without FCS. Thus the interaction of each type of UIF with FCS may produce different patterns of radioisotope uptake.

Similarly, with the thymocyte cultures, WCF.UIF produced more inhibition of radioisotope uptake than the other two UIFS. The inhibitory effects of the UIFS TC and RWC (within each batch) were relatively more variable than on the plasmacytoma cultures. TC.UIF (batch 19.2 cultured without FCS) was more inhibitory on the thymocytes than on plasmacytoma cultures, while batch 19.2 of RWC.UIF was more inhibitory (cultured without FCS) on plasmacytoma than on thymocytes.

The addition of FCS to TC.UIF (thymocyte cultures) produced a different pattern of radioisotope uptake at 20 and 40 ul: at 20 ul the uptake in the presence of FCS with batch 18.2 was similar to that without FCS, while the uptake in the presence of batch 19.2 and FCS was approximately reduced to half the value of the uptake for cells cultured without FCS, while the inhibition of radioisotope uptake by batch 29.2/FCS was greatly increased relative to the uptake by cultures cultured without FCS. At 40ul, the inhibition of radioisotope uptake was increased in the presence of FCS (different from the 20ul group) than for cultures cultured without FCS. The change in radioisotope uptake for batches 19.2 and 29.2 was similar to that of the 20ul group. For RWC.UIF, the general pattern of radioisotope uptake inhibition was higher in the presence of FCS than without FCS.

The inhibition of radioisotope uptake for WCF.UIF was nearly the same at both concentrations, slightly more inhibtion in the 20ul group cultured with FCS than without FCS. It can be noted that the thymocytes were more sensitive to the inhibitory effects of WCF.UIF (in the presence of FCS) than the plasmacytoma cultures. Thus the inhibition of radioisotope uptake by UIF on cell cultures depends on several factors, such as the presence of FCS, the type of UIF, the batch quality of UIF, the concentration of the UIF in the culture and the type of cell culture used for the assessment of UIF.

3.2.1.3: The effects of mouse UIF on DNA synthesis and the uptake of 125 IUDR by cell cultures.

Experimental procedures:

Several types of UIFS were prepared, from thymocytes, bone marrow cells, spleen cells (whole spleen UIF, RWC and white cell fraction WCF.UIF), and lymph node cells (inguinal, axillary and mesentric). Each UIF was prepared independently in tissue culture grade petri dishes (50 mm diameter sterilin) at a concentration of 1×10^7 cells/ml RPMI without FCS, with a total of 5×10^7 cells per petri dish. The cultures were incubated for 16 hours at 37°C in a Leec incubator. The thymocyte (TC.UIF), spleen (WCF and RWC.UIFS), bone marrow (BM.UIF) and lymph node (LN.UIF) batches are coded as A, B and C.

Suspensions of plasmacytoma cells and mouse thymocytes were prepared and each was dispensed into separate microtitre wells, at a concentration of 1×10^5 cells per well. Cell cultures were divided into two parts, those cultured with 10% FCS-HI and those cultured without FCS.

Each well received 20 ul or 40 ul of the respective UIF, together with 0.2 uci 125 IUDR. The final volume per well was 200 ul. Each UIF concentration was replicated in 5 wells. All cultures have control cultures, in which cells were cultured without UIF.

Results:

The difference in cpm between plasmacytoma cultures cultured without FCS (medium controls, 10343) and cultured with 10% FCS (FCS controls 7758) as described in Table 2.1.C1 was 24.99% due to FCS, and was significant ($t=4.192$ $P<.01$).

For cells cultured without FCS, the mean cpm for the respective

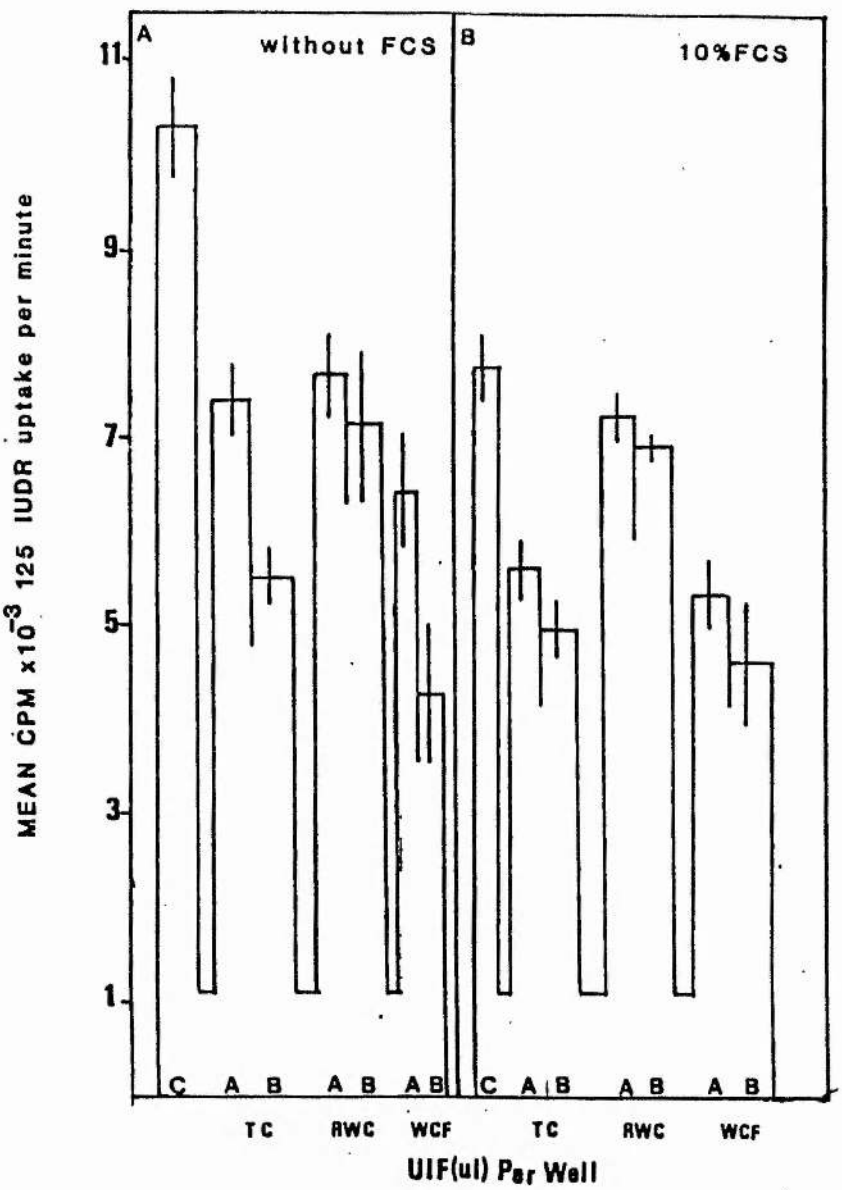


FIGURE 2.1.C1: The uptake of 125 IUDR by plasmacytoma cultures, cultured without and with FCS-HI, for 4 hours, with mouse UIF.
RWC: spleen red and white cells UIF. WCF: spleen white cell fraction.
TC: thymus cells UIF. LN: lymph nodes cells UIF.
BM: bone marrow cells UIF.

A: 20ul B: 40ul C: control

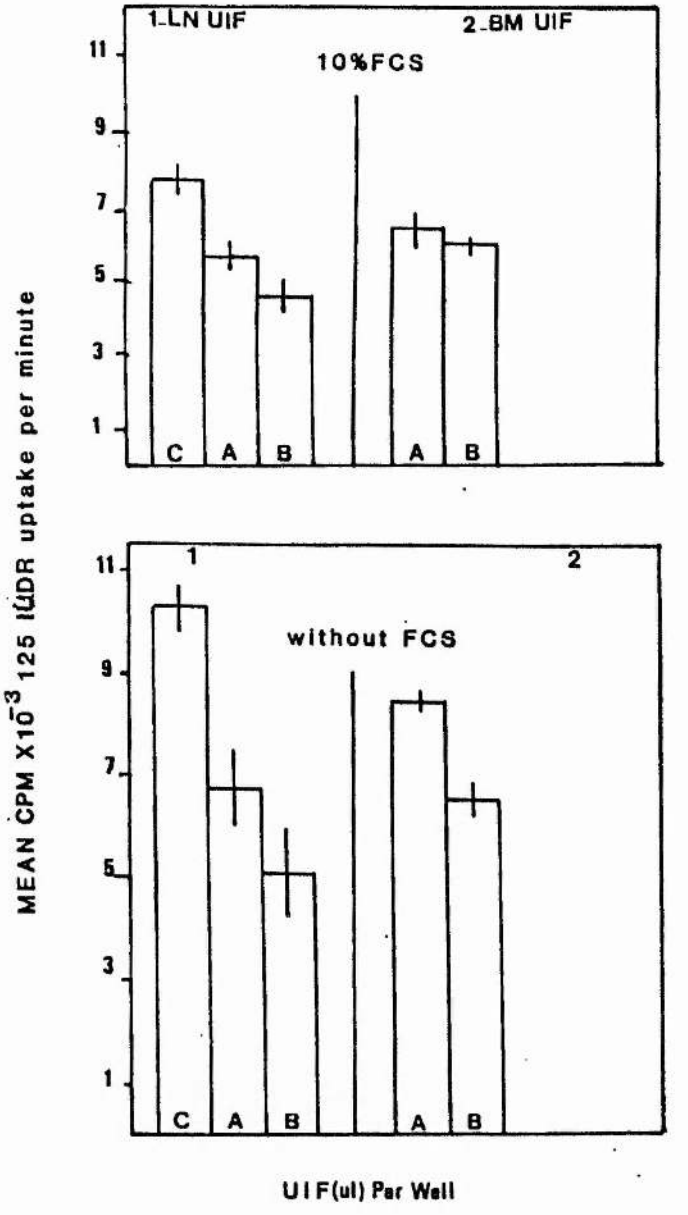


FIGURE 2.1.C1: The uptake of 125 IUDR by plasmacytoma cultures, cultured without and with FCS-HI, for 4 hours, with mouse UIF. RWC: spleen red and white cells UIF. WCF: spleen white cell fraction. TC: thymus cells UIF. LN: lymph nodes cells UIF. BM: bone marrow cells UIF.

Table 2.1.C1. The effects of mouse UIFS on the uptake of 125 IUDR by plasmacytoma cultures, cultured for 4 hours, with and without FCS. The values are expressed as mean cpm with ± S.E. included; together with a total mean cpm per UIF group of 3 batches, with its ± S.E. The respective percent inhibitions are included.

		Cultured without FCS		Cultured with 10% FCS	
IF type and concentration per well	UIF Batch	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
TC	A	7154 ± 204	30.8	5171 ± 356	33.3
20 ul	B	6955 ± 338	32.7	5469 ± 183	29.4
	C	8112 ± 245	21.5	6141 ± 371	20.8
Batch mean		7406 ± 357	28.33 ± 3.46	5593 ± 287	27.83 ± 3.69
TC	A	5108 ± 101	50.6	4596 ± 88	40.7
40 ul	B	5305 ± 152	48.7	4875 ± 144	37.1
	C	6077 ± 101	41.2	5452 ± 127	29.7
Batch mean		5497 ± 296	46.83 ± 2.87	4974 ± 252	35.83 ± 3.24
R&C	A	8602 ± 254	16.8	7628 ± 529	1.6
20 ul	B	7259 ± 156	29.8	7230 ± 432	6.7
	C	7255 ± 504	29.8	6846 ± 551	11.7
Batch mean		7705 ± 448	25.46 ± 4.33	7235 ± 225	6.66 ± 2.91
R&C	A	8640 ± 195	16.46	7155 ± 546	7.7
40 ul	B	6236 ± 162	39.7	6820 ± 144	12.08
	C	6520 ± 164	36.9	6876 ± 170	11.36
Batch mean		7132 ± 759	31.02 ± 7.33	6950 ± 103	10.36 ± 1.35

Table 2.1.C1: (continued)

UIF type and concentration per well	UIF Batch	Cultured without FCS		Cultured with 10% FCS	
		mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
Wcf	A	7180 ± 122	30.5	6115 ± 287	21.1
20 ul	B	6830 ± 372	33.95	4799 ± 395	38.1
	C	5250 ± 144	49.2	5190 ± 159	33.09
Batch mean		6420 ± 594	37.88 ± 5.75	5368 ± 390	30.76 ± 5.04
Wcf	A	5589 ± 299	45.9	5847 ± 66	24.6
40 ul	B	3987 ± 143	61.4	4298 ± 103	44.7
	C	3336 ± 149	67.7	3781 ± 142	51.2
Mean batch		4303 ± 670	58.33 ± 6.48	4639 ± 622	40.16 ± 8.01
LN	A	5331 ± 210	48.45	4674 ± 101	39.74
20 ul	B	7533 ± 285	27.16	5934 ± 240	23.50
	C	7396 ± 299	28.49	6187 ± 93	20.24
Batch mean		6753 ± 713	34.71 ± 6.91	5598 ± 468	27.82 ± 6.03
LN	A	3332 ± 279	67.78	3356 ± 155	56.73
40 ul	B	5873 ± 211	43.21	4950 ± 498	36.19
	C	5916 ± 131	42.8	5339 ± 75	31.17
Mean batch		5040 ± 855	51.26 ± 8.26	4548 ± 607	41.36 ± 7.82

Table 2.1.C1 (continued)

UIF type and concentration per well	UIF Batch	Cultured without FCS: mean cpm ± S.E.	Cultured without FCS: Percent Inhibition ± S.E.	Cultured with 10% FCS: mean cpm ± S.E.	Cultured with 10% FCS: Percent Inhibition ± S.E.
BM	A	8140 ± 284	21.29	5601 ± 323	27.79
20 ul	B	8736 ± 358	15.53	6860 ± 186	11.57
	C	8430 ± 146	18.49	6688 ± 108	13.79
Batch mean		8435 ± 172	18.43 ± 1.66	6383 ± 394	17.71 ± 5.08
BM	A	5895 ± 307	42.99	5746 ± 201	25.92
40 ul	B	6925 ± 300	33.03	6043 ± 154	22.1
	C	6702 ± 190	35.19	5906 ± 111	23.86
Batch mean		6507 ± 313	37.07 ± 3.02	5898 ± 85	23.96 ± 1.10
Medium Controls without FCS =		10343 ± 513	0.0	10% FCS Controls = 7758 ± 342	0.0

UIF.TC - Thymocytes UIF; UIF.RWC = Spleen : red and white cells UIF; UIF.Wcf = Spleen : white cell fraction UIF without red cells; UIF.LN = Lymphnode cells UIF; UIF.BM = Bone marrow cells UIF.

T-tests comparisons for Table 2.1.C1.

column 1 = UIF batch versus controls (without 10% FCS)
column 2 = UIF batch versus controls (with 10% FCS)

UIF Type		1	2
TC	A	p**	p**
20 ul	B	p**	p**
	C	p**	P*/*
	A	p**	p**
40 ul	B	p**	p**
	C	p**	p**
F&C	A	p*/*	p*
20 ul	B	p**	p*
	C	p**	p*
	A	p*/*	p*
40 ul	B	p**	p*/*
	C	p**	p*/ *
WcE	A	p**	p**
20 ul	B	p**	p**
	C	p**	p**
	A	p**	p**
40 ul	B	p**	p**
	C	p**	p**
LN	A	p**	p**
20 ul	B	p**	p**
	C	p**	p**
	A	p**	p**
40 ul	B	p**	p**
	C	p**	p**
BM	A	p**	p**
20 ul	B	p*/*	p*/*
	C	p**	p*/ *
	A	p**	p**
40 ul	B	p**	p**
	C	p**	p**

p*/* = .05 > P) .01
p** = <.01
p* = >.05

UIF batches of UIF.TC were all significantly different from the mean cpm of culture control ($p<.01$) as described in the t-test comparison table. The mean percent inhibition of isotope uptake at 20 ul UIF was 28% which increased to about 47% with 40 ul UIF per well. For UIF.RWC the cpm of the respective batches were all significantly different from control cultures, ($P<.01$) except for batch A (40 ul UIF) at ($0.05>P>.01$). The mean per cent inhibition produced by 20 ul UIF was 25% with a minor increase to 31% at 40 ul UIF per well. For UIF.WCE , the Cpm of the respective batches were all significantly different from culture controls, ($p<.01$) as detailed in the test table. At 20 ul UIF per well, there was about 38% inhibition of isotope uptake, which increased moderately to 58% at 40 ul UIF per well. The cpm for the respective batches of UIF.LN at 20 and 40 ul were all significantly different from culture control cpm, ($P<.01$). The mean percent inhibition at 20 ul UIF per well was around 35% with a fair increase to 51% at 40 ul UIF per well.

For UIF.BM similarly, the cpm of the respective batches were all significantly different from the cpm of control cultures, ($P<.01$), except for batch B (20 ul UIF) at ($0.05>P>.01$). The mean percent inhibition at 20 ul UIF per well was 18% which increased by a factor of 2 to 37% with 40 ul UIF per well.

An Analysis of Variance (Anovar.) for the 20 ul UIF per well between the mean percent inhibitions of all the batches of UIFS: TC, RWC, WCE, LN and BM, showed that they were not significantly different from each other, ($P>.05$, F.ratio=2.88). Similarly for the 40 ul per well group, the Anovar., showed no significant differences ($P>.05$ $F=3.33$).

For cells cultured with 10% FCS, the cpm of the respective batches of UIF.TC were significantly different from the cpm of

control cultures ($P<.01$) except for batch **C** (20 ul UIF) at ($0.05>P>.01$). At 20 ul UIF the mean percent inhibition of isotope uptake was around 28% with a small increase to 36% at 40 ul UIF per well. For UIF.RWC at 20 ul UIF per well the cpm of respective batches were not significantly different from cpm of culture controls, ($P>.05$). At 40 ul per well the cpm of batch A was not significantly different from culture controls, ($P>.05$), whereas batches B and C were different, ($0.05>P>.01$).

However, the total mean cpm for the three batches of UIF.RWC at 40 ul per well was not significantly different from culture controls, ($P>.05$, $t=2.268$).

At 20 ul per well, RWC.UIF produced a mean percent inhibition of 6.6% with a minor increase to 10% at 40 ul per well. For WCE.UIF , the mean percent inhibition at 20 ul UIF per well was around 31% which increased to 40% with 40 ul UIF per well.

The cpm of the respective batches of WCE.UIF at 20 ul or 40 ul per well were significantly different from the cpm of control cultures $P<.01$ as described in the t-test table.

The mean cpm of the respective batches of UIF.LN at 20 and 40 ul per well were all significantly different from the mean cpm of control cultures, $P<.01$. At 20 ul UIF per well, the mean percent inhibition was approximately 28% which increased moderately to 41% at 40 ul UIF per well.

The mean cpm for the respective batches of UIF.BM at 20 ul or 40 ul per well were all significantly different from the Cpm of control cultures ($P<.01$), except for batches B and C, 20 ul UIF, ($0.05>P>.01$). The mean percent inhibition produced by 20 ul UIF per well was 38% which increased to about 43% at 40 ul UIF per well. An Anovar between the mean percent inhibitions of all the batches of UIF: TC,

RWC, WCE, LN and BM were significant at 20 ul, (0.05>P>.01, F=4.53), and at 40 ul per well, (P<.01, F=6.19).

At 20 ul UIF, WCE, TC and LN-UIFS were different from RWC.UIF while BM.UIF was not different. UIFS: WCE, TC, LN and BM were not different among each other. At 40 ul per well, UIFS: LN, WCE and TC were different from RWC.UIF, while BM.UIF was not. UIFS: LN, WCE and BM were not different among each other. The differences among the UIFS were, assessed by using the multiple range test using short significant ranges (MRT-SSR).

For cells cultured without FCS and 20 ul UIF, the highest inhibition was produced by WCE 37.88%, followed by LN 34.71, TC 28.33, RWC 25.46 and BM 18.43. At 40 ul the highest inhibition was by WCE 58.33% followed by LN 51.26, TC 46.83, BM 37.07, and RWC 31.02%, as described in Table 2.1.C1 .

For cells cultured in 10% FCS with 20 ul UIF the highest inhibition was by WCE.UIF 30.76%, followed by TC and LN UIFS which were similar at 27.8%, BM 17.71% and RWC 6.66%. At 40 ul UIF the highest inhibition was by LN.UIF 41.36%, followed by WCE 40.16%, TC 35.83%, BM 23.96% and RWC 10.38%.

A comparison between the mean percent inhibitions caused by the three UIFS administered to cells, cultured with or without 10% FCS, using a 2tail t-test has been described, (Table 2.1.3)

The mean percent inhibitions for TC.UIF , 20 ul and 40 ul were not different between cultures containing FCS or without FCS. For the RWC.UIF at 20 and 40 uls, the mean percent inhibitions were higher for cells cultured without FCS than with FCS. The WCE.UIF , at 20 or 40 ul with or without FCS showed no difference in mean percent inhibitions, so was the case with LN.UIF .

The BM.UIF at 40 ul, was more inhibitory for cells cultured without FCS than with FCS, although at 20 ul there was no difference. The percent inhibition described in Table 2.1.C1 were determined as has been described in Table 2.1.B1.

Results:

. The difference in cpm between mouse thymocytes cultured without FCS (medium controls, 192 cpm) and cells cultured with 10% FCS (FCS controls, 186 cpm) as described in Table 2.1.C2 was 3.1% which was not significant t=0.197 P>.05 2tail.

For cell cultures, cultured without FCS, the batch total mean cpm for UIF.TC at 20 and 40 ul per well were significantly from the mean Cpm of the control culture, (P<.01, t=7.38 and 8.54). The corresponding mean percent inhibition at 20 ul per well was 63% which increased to 71.8% at 40 ul per well. For UIF.RWC the batch total mean cpm at 20 and 40 ul per well were significantly different from culture control, (0.05>P>.01 t=2.73), (P<.01, t=5.00) respectively. At 20 ul the batch total mean percent inhibition was 31.5%, which increased to 45% at 40 ul UIF per well. The batch total mean cpm of UIF.WCE at 20 and 40 ul per well were also significantly different from the mean Cpm of culture control (P<.01, t=3.44 and 4.916) respectively. The batch total mean percent inhibition at 20 ul per well was 42% which icnreased to 51% at 40 ul UIF per well.

An Anovar. comparison between the mean percent inhibitions for UIFS: TC, RWC and WCE at 20 ul (without FCS) showed that some differences existed (0.05>P>.01, F=8.78). TC.UIF was different from RWC.UIF but not from WCE.UIF , and RWC.UIF was not different from WCE.UIF . At 40 ul per well there were no significant differences among the mean percent inhibitions of the respective UIFS, (P>.05, F=2.497), and an MRT-SSR analysis.

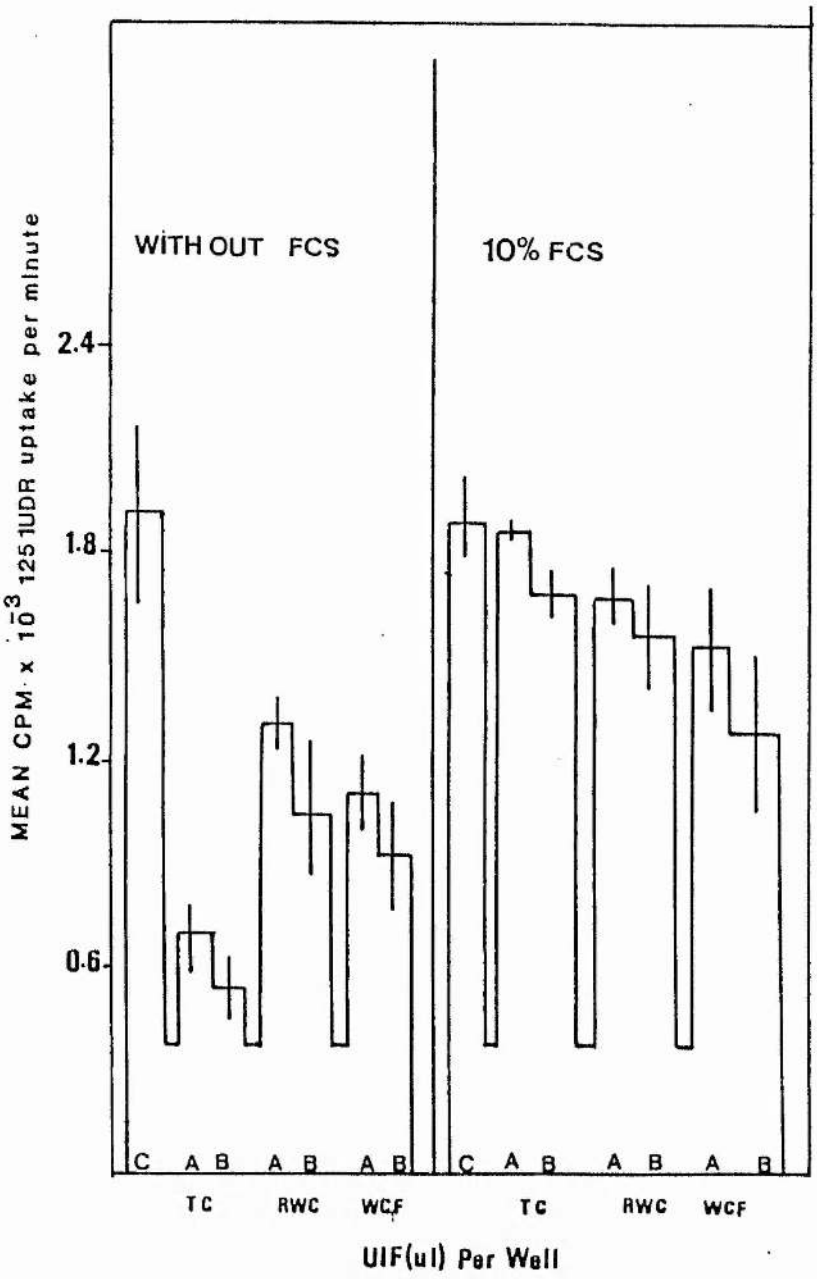


FIGURE 2.1.C2: The uptake of 125 IUDR by mouse thymocyte cultures, cultured without and with FCS-HI, for 4 hours, with mouse UIF.
RWC: spleen red and white cells UIF. WCF: spleen white cell fraction.
TC: thymus cells UIF.
A: 20ul B: 40ul C: control

Table 2.1.C2: The effects of Mouse UIFS on the uptake of 125IUDR by mouse thymus cells, cultured for 4 hours, with and without 10% FCS. The values are expressed as mean cpm with ± S.E. included; together with a total mean cpm per UIF group of 3 batches, with its ± S.E. The respective percent inhibitions are included.

		Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well.	UIF Batch	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
TC	A	90 ± 10	53.1	188 ± 17	0.5
20 ul	B	54 ± 8	71.8	189 ± 17	00.00
	C	67 ± 4	65.1	182 ± 16	3.7
Total mean		70 ± 10	63.3 ± 5.47	186 ± 2	1.4 ± 1.16
40 ul	A	70 ± 13	63.5	164 ± 14	13.2
	B	56 ± 5	70.8	157 ± 10	16.9
	C	35 ± 2	81.7	185 ± 8	2.1
Total mean		53 ± 10	71.83 ± 5.31	168 ± 8	10.7 ± 4.45
RWC	A	138 ± 27	28.1	187 ± 31	1.05
20 ul	B	140 ± 10	27	159 ± 25	15.8
	C	116 ± 12	39.5	155 ± 19	17.9
Total mean		131 ± 7	31.53 ± 4	167 ± 10	17.5 ± 0.93
40 ul	A	71 ± 11	63	185 ± 41	2.11
	B	147 ± 7	23.4	150 ± 21	20.6
	C	98 ± 9	48.9	134 ± 31	29.1
Total mean		105 ± 22	45.1 ± 11.6	156 ± 15	17.22 ± 7.97

Table 2.1.C2: (continued)

UIF type and concentration per well	UIF Batch	Cultured without FCS: mean cpm ± S.E.	Cultured without FCS: Percent Inhibiton ± S.E.	Cultured with 10% FCS: mean cpm ± S.E.	Cultured with 10% FCS: Percent Inhibition ± S.E.
Wcf	A	127 ± 18	33.8	186 ± 22	1.5
20 ul	B	86 ± 13	55.2	148 ± 8	21.6
	C	120 ± 23	37.5	124 ± 8	34.3
Total mean		111 ± 12	42.16 ± 6.61	152 ± 18	19.13 ± 9.55
40 ul	A	111 ± 15	42.1	166 ± 19	12.1
	B	110 ± 15	42.7	129 ± 11	31.7
	C	60 ± 10	68.7	90 ± 10	52.3
Total mean		93 ± 16	51.16 ± 8.77	128 ± 31	32.03 ± 11.6
Medium Control = 192 ± 27				10% FCS Control = 189 ± 31	

UIF.TC = Thymocytes UIF; UIF.RWC = Spleen : red and white cells UIF; UIF.Wcf = Spleen : white cell fraction UIF, without red cells.

For cells cultured with 10% FCS, the batch total mean percent inhibitions at 20 and 40 ul per well were small, at 1 to 10%, and the batch total mean cpm for both UIF concentrations were not significantly different from the cpm of culture control ($P>.05$, $t=0.161$ and 1.53 respectively). Similarly, for RWC.UIF the batch total mean cpm at both concentrations of UIF per well, were not significantly different from culture control ($P>.05$, $t=0.827$ and 1.016) respectively. The respective inhibitions at 20 and 40 ul UIF per well were similar at around 17%. For WCE.UIF , the batch total mean cpm for 20 and 40 ul were significantly different from controls, ($0.05>P>.01$, $t=2.429$, and $P<.01$, $t=3.89$), respectively. The batch total mean percent inhibition at 20 ul was 19% which increased fairly to 32% at 40 ul per well. An Anovar between the batch total mean percent inhibitions at 20 or 40 ul of UIFS TC, WRC and WCE, showed no significant differences ($P>.05$, $F=1.96$ and 1.63), respectively.

The highest inhibition of 125 IUDR uptake for cells cultured without FCS, was by TC.UIF at 20 ul: 63.3%, followed by WCE.UIF : 42.16% and RWC.UIF: 31.53% and at 40 ul TC.UIF produced 71.83% inhibition, followed by WCE 51.16% and RWC 45.1% inhibition.

For cells cultured with 10% FCS the highest inhibition was by WCE.UIF 20 ul and 40 ul: 19.13% and 32.03% respectively, followed by RWC.UIF : of 17.5% and 17.22% respectively and for TC.UIF : 1.4% and 10.7% at 20 ul and 40 ul respectively.

A comparison between the mean percent inhibitions caused by the 3 UIFS administered to cells, cultured with or without 10% FCS using 2tail t-test has been described, (Table 2.1.3).

Table 2.1.2: T-test comparisons.
20 & 40 ul.

UIF.FCS VS UIF WITHOUT FCS

TC:	20,	P*
	40,	P*
RWC:	20,	P*/*
	40,	P*/*
WCF:	20,	P*/*
	40,	P**

P*/* = .05>P>.01
P** = .01
P* = .05

Table 2.1.3: T-test comparisons.
20 & 40 ul.

UIF.FCS vs UIF WITHOUT FCS

		Pc	Tc
TC:	20,	P-	P**
	40,	P*	P**
RWC:	20,	P*/*	P-/-
	40,	P*/*	P*
WCF:	20,	P*	P-
	40,	P*	P*
LN:	20,	P*	
	40,	P*	
BM:	20,	P-	
	40,	P*/*	

Pc = plasmacytoma
Tc = thymocytes

The mean percent inhibition for TC.UIF in cultures without 10% FCS were significantly greater than their counterparts cultured with 10% FCS; at 20 ul and 40 ul.

For the RWC.UIF the mean percent inhibition was greater in cells cultured without FCS than with FCS $P<.05$ at 20 ul; while at 40 ul no differences existed.

For the WCE.UIF the mean percent inhibitions for cells cultured without FCS were not significantly different from their counterparts cultured with FCS.

The percent inhibitions described in Table 2.1.C2 were determined as has been described in Table 2.1.B1.

As described above, (section 3.2.1.3) the effects of several mouse UIFS on the uptake of radioisotope by plasmacytoma and mouse thymocytes were assessed.

For plasmacytoma cultures, the difference (within the respective batches) on the inhibition of radioisotope uptake were relatively less than that present among the rat UIF batches, (section 3.2.1.2).

Similarly, the inhibitory effects differed among the respective UIFS, and at 20ul UIF (without FCS) the highest inhibition was by WCF and LN UIFS followed by TC, RWC and the least inhibitory was BM UIF. At 40ul UIF (with FCS), the highest inhibition was by WCF, LN and TC UIFS (which were within a similar inhibitory range), while the least inhibitory was BM UIF. The relative inhibition of radioisotope uptake was modulated by FCS and the inhibitory effects of UIFS (without FCS) were all decreased by the presence of FCS, (except TC and BM 20ul) especially RWC UIF. It was also demonstrated that mouse TC UIF was more inhibitory than rat TC UIF described earlier, (section 3.2.1.2).

For the thymocytes, the variation in the inhibition of radioisotope uptake within each respective UIF group was small. The highest inhibition of uptake was by the TC group (20ul without FCS) followed by WCF and RWC UIFS. Similarly at 4o ul, the highest inhibition was by the TC group followed by WCF and RWC UIFS which were in the same inhibition range. This was not the case with FCS culture group, as the highest inhibition of radioisotope uptake was by WCF UIF followed by RWC and the least inhibitory was TC UIF. FCS has interacted with UIF in the presence of the thymocytes to produce this reverse in inhibition, which was not evident in the plasmacytoma cultures, (sections 3.2.1.2 and 1.3), but it was

rather similar to the effects demonstrated with rat TC UIF on mouse thymocytes, (section 3.2.1.2). As with rat UIF, FCS was demonstrated to interact selectively with each UIF group.

Mouse UIFS, were generally more inhibitory than rat UIF. The UIFS produced from cells from different haemopoietic environments (bone marrow, lymph nodes, spleen and thymus) were assessed in terms of their inhibitory effects on radioisotope uptake by 2 cell culture types, and were shown to be inhibitory to various degrees.

3.2.2: The effects of various types of rat UIF on the uptake of 125IUDR by cell cultures.

Experimental Procedures:

The following UIFS (1-7) were prepared from Rat spleens:

1. UIF prepared from cells of the spleen Wcf (white cells only and this is coded as STD, the standard UIF, to which other UIFS will be compared with, (STD.UIF).

2. UIF prepared from adherent cells of the spleen's (Wcf) white cell fraction, (AD.UIF).

3. UIF prepared from non-adherent cells of the spleen's (Wcf) white cell fraction, (NAD.UIF).

4. UIF prepared from macrophage free cells, of the spleen's (Wcf) white cell fraction, (φfree.UIF).

5. UIF prepared from cells of the spleen's white cell fraction (Wcf), but at 5×10^6 cells per ml (5M.UIF).

6. UIF prepared from the spleen's white cell fraction (Wcf) which was later dialysed, (DZ.UIF).

7. UIF prepared from the spleen's white cell fraction (Wcf) which had been frozen and thawed, (F.TH.UIF).

8. UIF prepared from the BC_3H-1 muscle cells; (B.UIF).

The procedure for the preparation of Wcf the standard UIF (STD) was by incubating 50×10^6 cells in 5 mls of RPMI in 50 mm diameter petri dishes, for 16 hours at 37°C. The detailed procedures for the preparations of all the UIFS listed above have been described in materials and methods.

Cell suspensions of plasmacytoma cells and of mouse thymocytes were prepared and dispensed into their respective microtitre wells, each at a concentration of 1×10^5 cells per well. Cell cultures were divided into two parts, those cultured without FCS, and those cultured with 10% FCS. Microtitre wells received 20 and 40 uls of each type of UIF, and 0.2 uci of 125 IUDR in a final volume of 200 ul per well. Each UIF concentration was replicated 5 times. The incubation periods were as follows: 0/4, 2/4 and 4/4 sequences.

In the 0/4 group, the UIF and 125 IUDR were added to cell cultures at zero time. The cell cultures were incubated for 4 hours at 37°C.

In the 2/4 group, the UIF was added to cell cultures at zero time, and incubated for 2 hours at 37°C. Following the 2 hour incubation, 125 IUDR was added to the cell cultures and re-incubated for 4 hours at 37°C.

In the 4/4 group, the UIF was added to cell cultures at zero time and incubated for 4 hours at 37°C. Following the 4 hours incubation, 125 IUDR was added to cell cultures and re-incubated for 4 hours.

All cultures have their controls, in which cells were cultured without UIF.

Results:

The mean cpm of plasmacytoma cultures cultured without 10% FCS was (medium controls) 13696 cpm , and with 10% FCS was (FCS controls) 6588 cpm , as described in Table 2.2.A1, (incubation sequence 0/4). The difference between the respective control cultures was significant ($P<.01$, $t=19.528$) which represented a 51.9% inhibition due to serum.

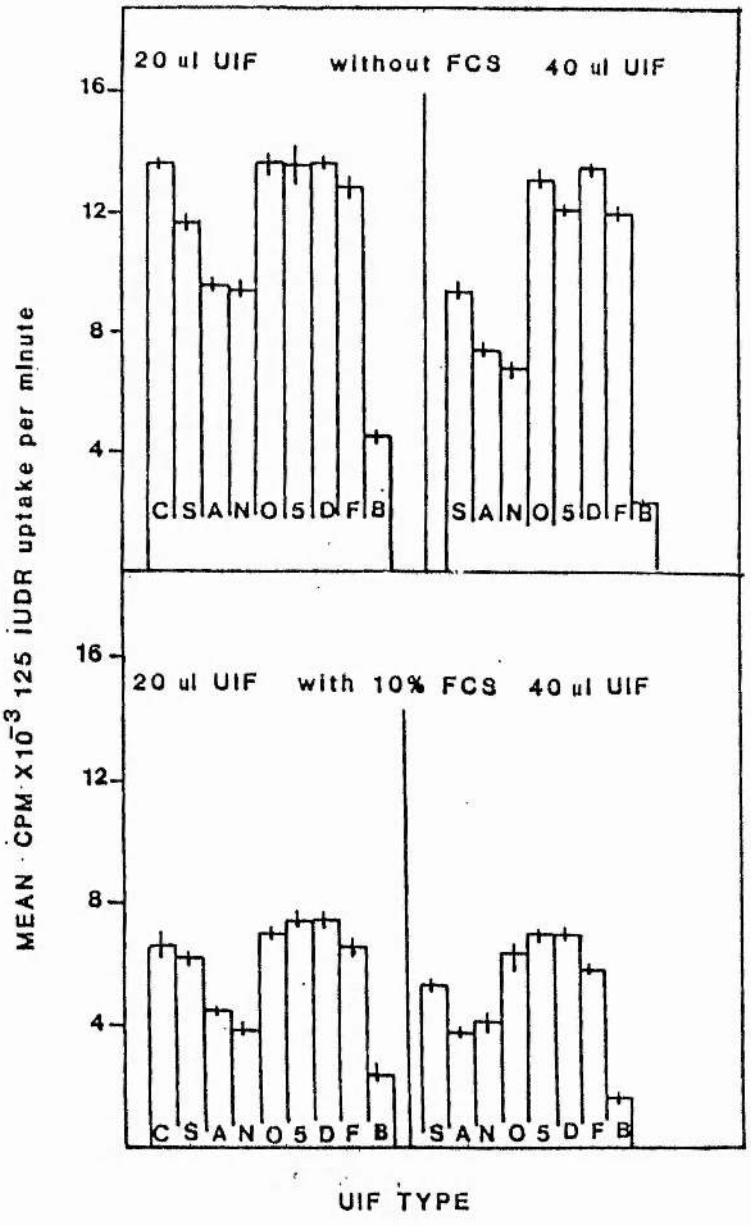


FIGURE 2.2.A1: The uptake of 125IUDR by plasmacytoma cultures, cultured without and with FCS-HI, for 4 hours, with rat UIF. 0/4 incubation sequence. C: control cultures without UIF. S: standard UIF. N: non-adherent cells UIF. A: adherent cells UIF. O: macrophage free UIF. 5: standard UIF, produced at half its culture density. D: dialysed UIF. F: freeze thaw UIF. B: muscle cell UIF

Table 2.2.A1: The effects of several types of Rat UIF on the uptake of 125IUDR by plasmacytoma cultures cultured with or without 10% FCS for a 0/4 hour incubation period. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± S.E. is included.

	Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
20 ul				
STD	11666 ± 191	14.8 ± 1.4	6247 ± 211	5.17 ± 3.21
AD	9616 ± 268	29.8 ± 1.95	4451 ± 142	32.4 ± 2.16
NAD	9475 ± 229	30.8 ± 1.66	3844 ± 315	41.6 ± 4.78
ϕ Free	13684 ± 342	0.09 ± 2.5	6958 ± 184	+5.62 ± 2.8
5M	13603 ± 495	0.67 ± 3.6	7363 ± 234	+11 ± 3.55
DZ	13628 ± 189	0.5 ± 1.36	7384 ± 119	+12 ± 1.8
FTH	12847 ± 299	6.1 ± 2.2	6537 ± 166	0.7 ± 2.52
B	4538 ± 158	66.8 ± 1	2381 ± 207	63.8 ± 3.14
40 ul				
STD	9358 ± 183	31.6 ± 1.34	5277 ± 61	19.89 ± 1
AD	7381 ± 189	46.1 ± 1.38	3725 ± 26	43.44 ± 0
NAD	6811 ± 129	50.2 ± 1	4109 ± 312	37.62 ± 4.73
ϕ Free	13055 ± 179	4.67 ± 1.3	6274 ± 372	4.75 ± 5.65
5M	12070 ± 161	11.8 ± 1	6878 ± 119	+4.4 ± 1.82
DZ	13501 ± 150	1.4 ± 1	6895 ± 84	+4.6 ± 1.3
FTH	11976 ± 181	12.5 ± 1.32	5736 ± 66	12.9 ± 1
B	2267 ± 38	83.4 ± 0	1607 ± 43	75.6 ± 0
Controls	13696 ± 133	0.0	6588 ± 338	0.0

+ = stimulation of uptake. STD UIF = Standard UIF; AD = Adherent cells of STD UIF; NAD = Non adherent cells of STD UIF; ϕ Free = Macrophage Free STD UIF; 5M = STD UIF at half culture density; DZ = dialysed STD UIF; FTH = freeze thawed STD UIF; B = Bc_3H-1 muscle cell line UIF.

For cells cultured without FCS, the mean percent inhibitions of 125 IUDR uptake by 20 ul of UIF, were similar for NAD and AD.UIFS at approximately 30%, which were the highest in the spleen group followed by STD.UIF at approximately 15%, and all their respective cpm were significantly different from their control cultures mean cpm, ($P<.01$, $t=15.93$, 13.63 and 8.716 respectively).

The least inhibitory were: ϕfree.UIF (0.09%), DZ.UIF (0.5%), 5M.UIF (0.67%) and F.TH.UIF (6.1%) of which, the mean cpm of the first three UIFS were not significantly different from the mean cpm of their control cultures, ($P>.05$, $t=0.092$, 0.294, 0.181) respectively, while the mean cpm of cultures, cultured with F.TH.UIF was signficant ($0.05>P>.01$). The B.UIF produced the highest inhibition of 66.8%, with its mean cpm was significantly different from the mean cpm of control cultures,($P<.01$, $t=44.3$).

Adding 40 ul of UIF (for cells cultured without FCS) the mean percent inhibitions increased relative to the 20 ul described above. NAD and AD UIFS were of similar strength at 50 to 46% inhibition, followed by STD.UIF at 31.6% inhibition. The respective mean cpm of these 3 UIFS was significantly different from the mean cpm of their control cultures ($P<.01$, $t=37.12$, 27.25 and 19.14) respectively. The least inhibitory were DZ.UIF (1.4%), ϕfree.UIF (4.67%), 5M.UIF (11.8%) and F.TH.UIF (12.5%). The mean cpm of cultures, cultured with DZ.UIF was not significantly different from the mean cpm of culture controls ($P>.05$, $t=0.975$); the mean cpm of cultures with ϕfree.UIF was significantly different from the mean cpm of culture controls ($0.05>P>.01$, $t=2.86$) while the mean cpm of 5M.UIF and F.TH.UIF were also significantly different from the mean cpm of controls ($P<.01$, $t=7.77$ and 7.65 respectively). The B.UIF produced the highest inhibition of 83.4%,($P<.01$, $t=82.4$).

Thus for 20 ul UIF cultured without FCS the relative inhibitory activities of all UIFS in terms of STD.UIF were as follows:-

The highest inhibition in the spleen group was produced by NAD and AD.UIFS which were in the region of 18 - 17% and were significantly different from STD.UIF, ($P<.01$, $t=7.34$ and 6.23) respectively. The other UIFS: (ϕfree, 5M, DZ) were similar in their stimulation of isotope uptake, and were all in the region of +17%, while F.TH stimulated the uptake by 10.12%. All stimulations of uptake were significantly different from STD.UIF; $P<.01$, except F.TH.UIF, $0.05>P>.01$. The t-test values were 5.14, 3.65, 7.29 and 3.32 respectively. B.UIF produced the highest inhibition of 61.1% relative to STD.UIF, which was signficantly different, ($P<.01$, $t=28.75$). With 40 ul of UIF (cells cultured without serum) the relative mean percent inhibitions relative to STD.UIF were higher than in 20 ul UIF. NAD.UIF produced 27.21% inhibition of isotope uptake, and AD produced 21.12%. The stimulatory activities of F.TH and 5M UIFS were similar, and were around 28%, while ϕfree and DZ UIFS were more stimulatory and both were in the region of 40%. The stimulatory activity of 40 ul UIF was higher than that of 20 ul UIF. The highest inhibition at 40 ul of UIF was produced by B.UIF, which was 75.77% and its respective mean cpm was significantly different from the mean cpm of culture controls i.e. STD.UIF cultures ($P<.01$, $t=37.88$). The F.TH, 5M, ϕfree and DZ UIFS stimulatory activities were all significantly different from control, ($P<.01$, $t=10.16$, 11.10, 14.40 and 17.49 respectively).

UIF used in conjunction with plasmacytoma cultured in 10% FCS has also depressed 125 IUDR uptake.

At 20 ul UIF per well, NAD.UIF produced the highest inhibition

in the spleen group which was 41.6%, followed by AD.UIF at 32.4%, and the least inhibitory were STD.UIF at 5.17% and F.TH.UIF at 0.7%. The mean cpm of cell cultures cultured with NAD and AD UIFS were significantly different from their control cultures mean cpm ($P<.01$, $t=5.937$ and 5.822 respectively), while the mean cpm of cultures with STD and F.TH UIFS were not, ($P>.05$, $t=0.853$ and 0.135).

The effects of ϕfree, 5M and DZ UIFS were all stimulatory with increased 125 IUDR uptake of +5.62%, +11.76% and +12% respectively, although their respective mean cpm values were not significantly different from the mean cpm of 10% FCS control cultures, ($P>.05$, $t=0.962$, 1.882 and 2.21 respectively). At 40 ul UIF per well, there was more inhibition of 125 IUDR uptake, with AD.UIF producing 43.44% inhibition, NAD.UIF 37.62%, STD.UIF 19.89%, F.TH 12.9% while ϕfree.UIF was the least inhibitory at 4.75%. The mean cpm of cultures, cultured with AD, NAD, STD and F.TH UIFS were significantly different from the mean cpm of cell culture controls ($P<.01$, $t=8.420$, 5.392 and 3.809), respectively; ($F.TH\ 0.05>P>.01$, $t=2.465$), while ϕfree was not, ($P>.05$, $t=0.622$). 5M and DZ UIFS were similar in their stimulation of isotope uptake at around 4% and were less stimulatory than at 20 ul UIF. The mean cpm of cell cultures cultured with UIFS: 5M and DZ were not significantly different from the mean cpm of culture controls ($P>.05$, $t=0.807$ and 0.879) respectively. B.UIF produced the highest inhibition of 75.6%, and its mean cpm was significantly different from the mean cpm of culture controls ($P<.01$, $t=14.585$). The relative inhibitions or stimulations of 20 ul and 40 ul UIFS cultured with 10% FCS in terms of STD.UIF are described in a t-test table, comparison, (Table 2.2.A).

Thus the effects of all the respective UIFS on isotope uptake by cell cultures were significantly different from STD.UIF, as AD, NAD and B UIFS were inhibitory while φfree, 5M, DZ and F.TH were stimulatory. Comparing the mean percent inhibitions produced by the respective UIFS (20 ul and 40 ul) between cells cultured without and with 10% FCS have shown that at 20 ul UIF, STD.UIF was less inhibitory with FCS than in cultures without FCS. AD and NAD UIFS were more inhibitory with FCS than without FCS. F.TH was less inhibitory with FCS than without FCS, and φfree, 5M, DZ UIFS were all stimulatory, where as without FCS they were slightly inhibitory. At 40 ul of UIF, STD.UIF was less inhibitory with FCS than without FCS; AD.UIF produced approximate inhibitions with or without FCS, NAD was less inhibitory in FCS, the inhibition produced by φfree UIF was similar in both with and without FCS; 5M and DZ were stimulatory with FCS compared to some inhibition, without FCS. F.TH was similar in inhibitions with and without FCS. B.UIF produced similar inhibitions at 20 ul, while at 40 ul, the inhibitions with FCS were slightly less than without FCS. A t-test table,(Table 2.2.B) . shows the significance of the difference between inhibitions or stimulations, with and without 10% FCS, at 20 ul and 40 ul of UIF. (T-test: mean percent inhibition (without FCS) VS mean percent inhibition with 10% FCS).

Thus at 20 ul UIF all comparisons were not significant except for STD and DZ UIFS which were significant. AT 40 ul all comparisons were significant except for AD, φfree and F.TH UIFS which were not.

The effects of the respective UIFS on cell viability are described in Table 2.2.C0.

Results:

The mean cpm of of plasmacytoma cultures cultured without FCS was (medium controls) 15305 cpm , and with 10% FCS controls was (FCS controls) 9011 cpm as described in Table 2.2.A2, (incubation sequence 2/4) which was a difference of 41.12% inhibition of 125 IUDR due to FCS, and which was significant ($P<.01$, $t=17.29$).

The mean percent inhibitions of isotope uptake by cell cultures produced by 20 ul and 40 ul UIF (without FCS) were as follows: At 20 ul of UIF, AD and NAD produced similar inhibitions of 30 to 28% which were the highest inhibitions in the spleen group, followed by STD.UIF at 15%. The least inhibitory were F.TH (5.28%), 5M.UIF (1.29%) and ϕfree.UIF (0.27%). DZ.UIF was +2.52% stimulatory. The highest inhibition was produced by B.UIF (71.07%). At 40 ul UIF the mean percent inhibitions were higher than at 20 ul UIF. Similarly NAD and AD UIFS were the highest inhibitors in the spleen group at 51 to 49%, while STD.UIF inhibited the isotope uptake by 32%. Smaller inhibitions were produced by F.TH.UIF (17.72%), 5M (9.06%) and ϕfree (6.28%), while DZ.UIF was +1.39% stimulatory. The highest inhibition was produced by B.UIF (85.44%). The significance of the mean percent inhibitions of 20 ul and 40 ul of the respective UIFs in terms of their cpm, relative to the mean cpm of culture controls is described, (Table2.2.C).

Thus all comparisons were significant except for ϕfree and 5M UIFS at 20 ul, while for DZ.UIF at both concentrations.

The relative inhibitions of the respective UIFS in terms of STD.UIF and their significance are described in a t-test comparison.

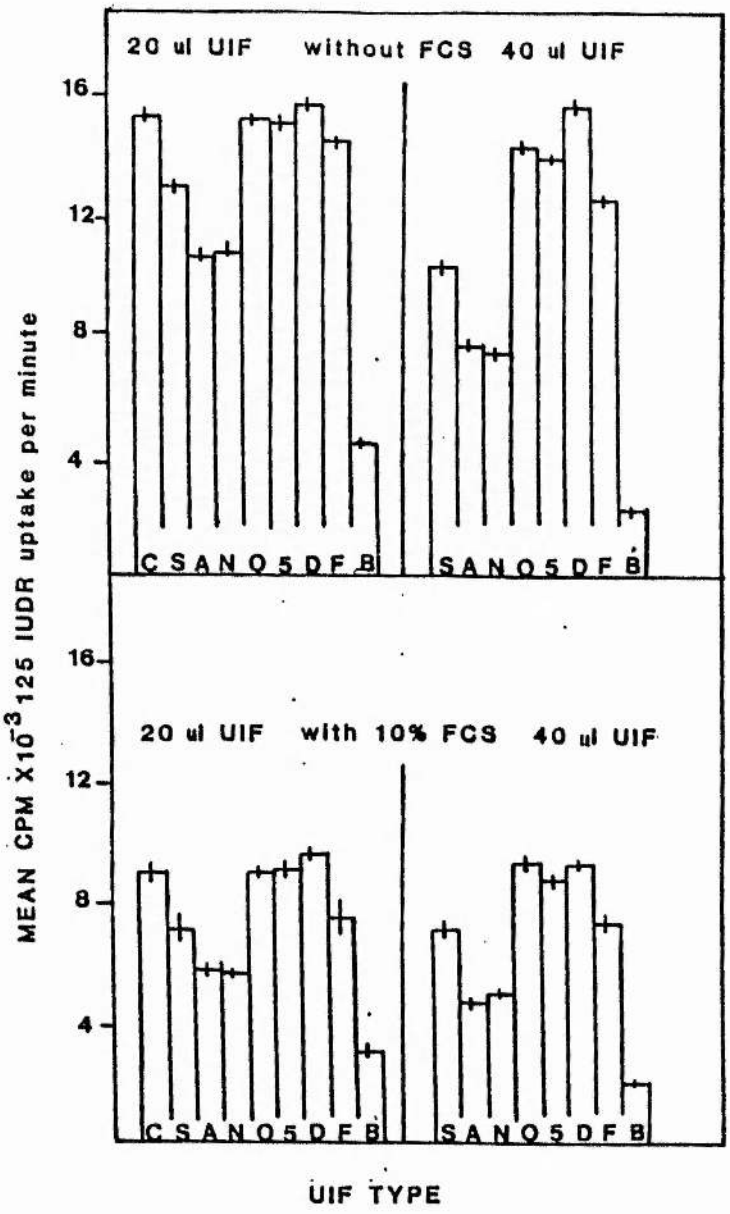


FIGURE 2.2.A2: The uptake of 125IUDR by plasmacytoma cultures, cultured without and with FCS-HI , for 4 hours with rat UIF. 2/4 incubation sequence. The UIF types are described in Fig. 2.2.A1 .

Table 2.2.A2: The effects of several types of Rat IUF on the uptake of 125IUDR by plasmacytoma cell cultures, cultured with our without 10% FCS for a 2/4 hour incubation period. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± S.E. is included.

	Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean ± S.E.	Percent Inhibition ± S.E.
STD 20 ul	13004 ± 163	15.03 ± 1	7315 ± 389	18.82 ± 4.32
AD	10707 ± 140	30.03 ± 0	5776 ± 161	35.89 ± 1.8
NAD	10908 ± 94	28.72 ± 94	5739 ± 115	36.31 ± 1.3
ø Free	15262 ± 120	0.27 ± 0	9059 ± 187	+0.53 ± 2
5M	15106 ± 248	1.29 ± 1.62	9152 ± 288	+1.56 ± 3.18
DZ	1569 ± 189	+2.52 ± 1.23	9620 ± 112	+6.76 ± 1.24
FTH	14496 ± 144	5.28 ± 1	7528 ± 549	16.45 ± 6.1
B	4227 ± 68	71.07 ± 0	3060 ± 132	66.04 ± 1.5
40 ul				
STD	10379 ± 3030	32.18 ± 1.98	7089 ± 76	21.32 ± 1
AD	7705 ± 110	49.65 ± 0	4608 ± 62	48.86 ± 0
NAD	7464 ± 97	51.22 ± 0	4947 ± 62	45.09 ± 0
ø Free	14343 ± 140	6.28 ± 1	9287 ± 276	±3.06 ± 3.06
5M	13917 ± 152	9.06 ± 1	8681 ± 175	2.66 ± 2
DZ	15519 ± 201	+1.39 ± 1.31	9170 ± 82	+1.76 ± 1
FTH	12592 ± 116	17.72 ± 0	7213 ± 216	19.95 ± 2.4
B	2227 ± 30	85.44 ± 0	1902 ± 48	78.89 ± 0
Control	15305 ± 235		9011 ± 277	

+ = stimulation of uptake; STD = Standard UIF; AD = Adherent cells of STD UIF; NAD = Non-adherent cells of STD UIF; ø Free = macrophage free UIF; 5M = STD UIF at half culture density; DZ = dialysed STD UIF; FTH = Freeze thawed STD UIF; B = BC_3H-1 muscle cell line UIF.

At 20 ul UIF, AD and NAD were relatively more inhibitory than STD.UIF, while φfree, 5M, DZ and F.TH were stimulatory rather than inhibitory. B.UIF was more inhibitory than STD.

At 40 ul UIF, AD and NAD UIFS were more inhibitory that STD.UIF, while φfree, 5M, DZ and F.TH were all stimulatory. The highest inhibition was produced by B.UIF.

The significance of the inhibitory or stimulatory activities of the repective UIFS in terms of their respective mean cpm, relative to the mean cpm of STD.UIF are as follows in the t-test comparison of the respective UIFS VS STD.UIF,(Table 2.2.D) Thus all comparisons were significant.

The mean percent inhibition for 20 and 40 ul UIF (with 10% FCS) were as follows: At 20 ul UIF, NAD and AD UIFS produced similar inhibitions of isotope uptake by cell cultures of 36 to 35%, while STD and F.TH UIFS were half as strong, inhibiting uptake by 18 to 16% respectively.

UIFS, φfree, 5M and DZ produced stimulation of 125 IUDR uptake of +0.53%, +1.56% and +6.76% respectively. The highest inhibition of 125 IUDR uptake of 66.04% was produced by B.UIF. At 40 ul of UIF there was more inhibition of isotope uptake by NAD and AD UIFS at 48 to 45% respectively, followed by STD and F.TH UIF at 21 to 19%, while the least inhibitory was 5M.UIF at around 3 to 4%. φfree and DZ UIFS produced stimulations of 125 IUDR uptake of +3.06 and 1.76 respectively. The highest inhibition was by B.UIF of 78.89%.

The significance of the inhibitory or stimulatory activities of 20 and 40 ul of UIF (with 10% FCS) in terms of their cpm relative to the mean cpm of their culture controls (10% FCS) is described in a t-test comparison, (Table 2.2.E).

Thus all comparisons were significant except φfree, 5M and DZ UIFS which were not at both UIF concentrations.

The relative inhibitions or stimulations of the respective UIFS in terms of STD.UIF are described in a t-test comparison below. At 20 ul UIF, AD and NAD UIFS were more inhibitory than STD.UIF. φfree, 5M, DZ and F.TH UIFS were all stimulatory compared to STD.UIF. The highest inhibition relative to STD.UIF was produced by B.UIF at 20 and 40 uls.

The significance of the inhibitions or stimulations of the respective UIFS (20 and 40 uls) in terms of their cultures mean cpm, relative to the mean cpm of STD.UIF cultures are described in a t-test comparison, (Table 2.2.F).

Thus all comparisons were significant except for F.TH.UIF at both concentrations which was not.

Comparing the mean percent inhibitions or stimulations produced by the respective UIFS (20 ul and 40 ul) between cells cultured with and without 10% FCS have shown that at 20 ul of UIF, the mean cpm of cultures cultured with STD, AD, NAD and F.TH UIFS were more inhibitory with 10% FCS than without FCS. B.UIF was less inhibitory with 10% FCS than without FCS. φfree, 5M, DZ were more stimulatory with 10% FCS than without FCS. At 40 ul of UIF, the mean cpm of cultures cultured with STD, AD, NAD, 5M and B UIFS were relatively less inhibitory with 10% FCS than their corresponding counterparts cultured without FCS. F.TH.UIF was more inhibitory with 10% FCS than without FCS. φfree and DZ UIFS were more stimulatory with FCS than without FCS. A t-test comparison, (Table 2.2.G) shows the significance of the difference between the mean inhibitions or stimulations with 10% FCS, or without FCS.

T-test comparison tables

Table 2.2.A:

UIF type VS STD UIF

	20 ul	40 ul
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P*/*	P*/*
5M:	P**	P**
DZ:	P**	P**
FTH:	P*	P**
B:	P**	P**

Table 2.2.B:

UIF.FCS VS UIF without FCS

	20 ul	40 ul
STD:	P*/*	P**
AD:	P*	P*
NAD:	P*	P*/*
ϕFr:	P*	P*
5M:	P*	P**
DZ:	P**	P**
FTH:	P*	P*
B:	P*	P**

Table 2.2.C:

UIF cpm VS Control cpm (without FCS)

	20 ul	40 ul
STD:	P**	P**
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P*	P**
5M:	P*	P**
DZ:	P*	P*
FTH:	P**	P**
B:	P**	P**

Table 2.2.D

UIF type VS STD UIF (without FCS)

	20 ul	40 ul
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P**	P**
5M:	P**	P**
DZ:	P**	P**
FTH:	P**	P**
B:	P**	P**

Table 2.2.E

UIF cpm VS Control cpm (10% FCS)

	20 ul	40 ul
STD:	P**	P**
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P*	P*
5M:	P*	P*
DZ:	P*	P*
FTH:	P*/*	P**
B:	P**	P**

Table 2.2.F

UIF type VS STD UIF (10% FCS)

	20 ul	40 ul
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P**	P**
5M:	P**	P**
DZ:	P**	P**
FTH:	P*	P*
B:	P**	P**

Table 2.2.G

UIF.FCS VS UIF without FCS

	20 ul	40 ul
STD:	P*	P**
AD:	P*/*	P*
NAD:	P**	P**
ϕFr:	P*	P*/*
5M:	P*	P*/*
DZ:	P*/*	P*
FTH:	P*	P*
B:	P*	P**

P*/* = .05 > P > .01
P** = <.01
P* = >.05

Thus at 20 ul UIF, STD, φfree, 5M, F.TH and B UIFS were not significantly different from their counterparts, while AD, NAD and DZ were different.

At 40 ul all UIFS were significantly different except for AD, DZ, and F.TH UIFS which were not.

Results:

The mean cpm for 125 IUDR uptake by plasmacytoma cultures, cultured without FCS was (medium controls) 14724 cpm , and with 10% FCS was (FCS controls) 11228 cpm , as described in (Table 2.2.A3, incubation sequence 4/4). There was a difference of 23.74% inhibition of 125 IUDR uptake due to FCS, which was significant, ($P<.01$, $t=10.748$).

The mean percent inhibitions or stimulations of isotope uptake by cell cultures, produced by 20 ul and 40 ul UIF (without FCS) were as follows: At 20 ul UIF, AD and NAD produced the highest inhibition of isotope uptake in the spleen group at 33 to 31% respectively, while STD.UIF was less inhibitory at 11% inhibition. φfree, 5M, DZ and F.TH produced a stimulation of isotope uptake of +4.23%, +6.06%, +3.53%, and 11.34% respectively. The highest inhibition of 76.18% was produced by B.UIF. At 40 ul UIF, AD, NAD, STD, F.TH, 5M, φfree and B UIF were relatively more inhibitory than at 20 ul, while DZ stimulations were similar, as described in Table 2.2.A3 .

The significance of the inhibitions or stimulations produced by the respective UIFS at 20 and 40 ul in terms of their cpm relative to the mean cpm of control cultures are described in a t-test comparison, (Table 2.2.H). Thus at 20 and 40 ul, the mean cpm of all UIF cultures were significantly different from their culture controls mean cpm except for φfree, 5M and DZ UIFS which were not.

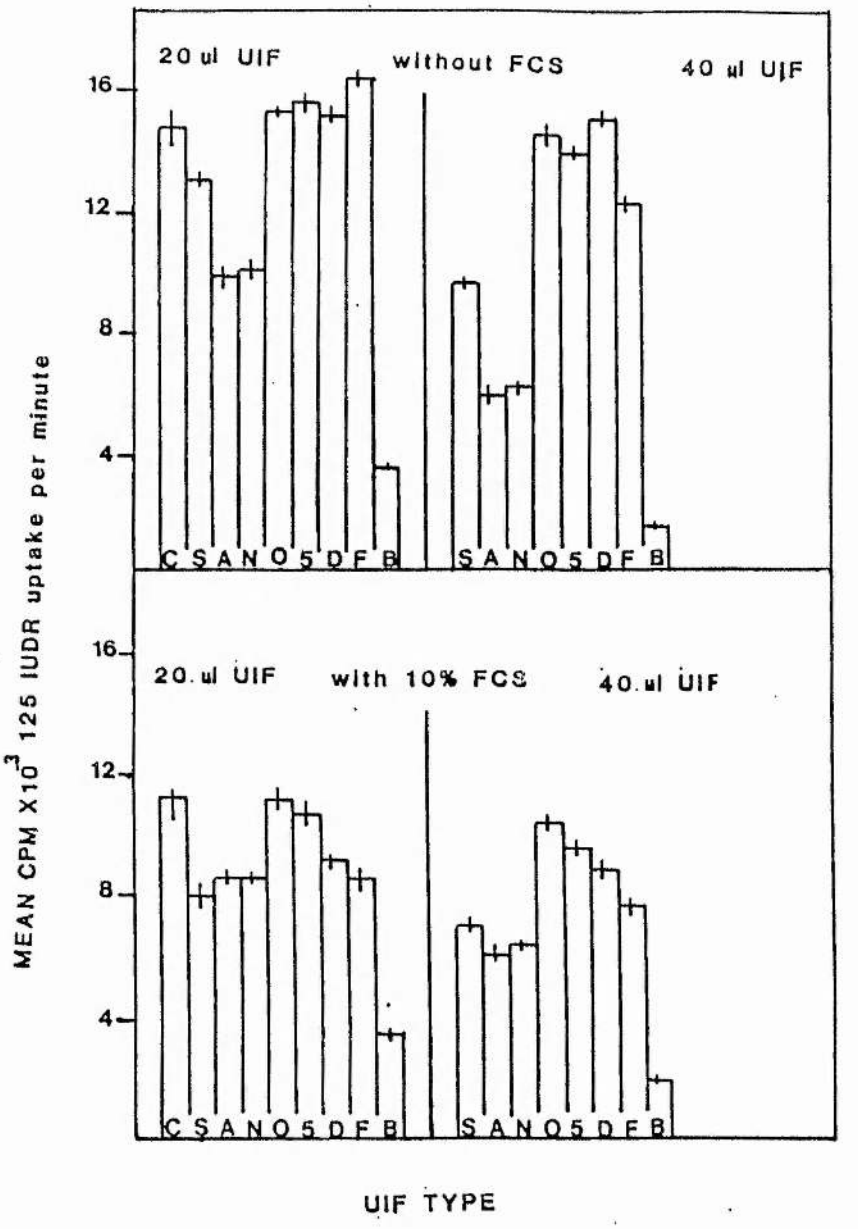


FIGURE 2.2.A3: The uptake of 125IUDR by plasmacytoma cultures, cultured without and with FCS-HI, for 4 hours with rat UIF. 4/4 incubation sequence. The UIF types are described in Fig. 2.2.A1.

Table 2.2.A3: The effects of several types of Rat UIF on the uptake of 125IUDR by plasmacytoma cell cultures, cultured with or without 10% FCS for a 4/4 hour incubation period. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± S.E. is included.

	Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
20 ul				
STD	13053 ± 342	11.34 ± 2.32	8051 ± 425	28.29 ± 1
AD	9751 ± 186	33.77 ± 1.3	8608 ± 184	23.33 ± 1.64
NAD	10114 ± 179	31.30 ± 1.3	8579 ± 201	23.59 ± 1.78
ø Free	15348 ± 118	+4.23 ± 1	11141 ± 643	0.77 ± 5.72
5M	15617 ± 339	+6.06 ± 2.3	10763 ± 493	4.14 ± 4.38
DZ	15245 ± 373	+3.53 ± 2.54	9184 ± 297	18.20 ± 2.65
FTH	16395 ± 226	+11.34 ± 1.54	8552 ± 422	23.82 ± 3.76
B	3506 ± 32	76.18 ± 0	3547 ± 141	68.40 ± 1.3
40 ul				
STD	9753 ± 195	33.75 ± 1.32	6985 ± 142	37.79 ± 1.3
AD	5965 ± 215	59.48 ± 1.46	6025 ± 131	46.33 ± 1.2
NAD	6256 ± 234	57.51 ± 1.6	6359 ± 81	43.36 ± 0
ø Free	14553 ± 431	1.16 ± 2.92	10335 ± 163	7.95 ± 1.45
5M	13977 ± 270	5.07 ± 1.83	9591 ± 218	14.58 ± 2
DZ	15120 ± 248	+2.68 ± 1.68	8803 ± 409	21.60 ± 3.63
FTH	12344 ± 247	16.16 ± 1.67	7661 ± 173	31.77 ± 1.54
B	1598 ± 50	89.14 ± 0	1994 ± 98	82.23 ± 1
Control	14724 ± 611		11228 ± 394	

+ = stimulation of isotope uptake. STD = Standard UIF; AD = Adherent cells of STD UIF; NAD = Non-adherent cells of STD UIF; ø Free = Macrophage free STD UIF; 5M = STD UIF at half culture density; DZ = Dialysed STD UIF; FTH = freeze thawed STD UIF; B = BC_3H-1 muscle cell line UIF.

The mean percent inhibitions of isotope uptake by cell cultures produced by 20 ul and 40 ul UIF with 10% FCS were as follows: At 20 ul of UIF, all UIFS were inhibitory. STD.UIF produced the highest inhibition in the spleen group of 28%, followed by AD, NAD and F.TH UIFS which were similar at 23% inhibition. DZ.UIF was 13% inhibitory while 5M and ϕfree were the least inhibitory at 4 and 0.77% respectively. B.UIF produced the highest inhibition at 68%. At 40 ul, all UIFS were more inhibitory than at 20 ul, the highest inhibitions in the spleen group were produced by AD and NAD UIFS at 46 to 43% respectively, followed by STD and F.TH UIFS at 37 to 31% respectively, while DZ produced 21% inhibition. The least inhibitory were 5M and ϕfree UIF at 14 to 7.95% respectively. The significance of the inhibitions produced by the respective UIFS at 20 or 40 ul in terms of their cultures mean cpm, relative to the mean cpm of control cultures are described in a t-test comparison, (Table 2.2.I). Thus at 20 and 40 ul all UIFS were significantly different from their controls except for ϕfree at both concentrations, and for 5M at 20 ul.

The relative mean percent inhibitions of the respective UIFS in terms of STD.UIF (20 and 40 uls) without FCS as described in Table 2.2.A3 were as follows: At 20 and 40 ul, AD and NAD UIFS were more inhibitory than STD.UIF, while ϕfree, 5M, DZ and F.TH UIFS were more stimulatory and not inhibitory as compared to STD.UIF. B.UIF produced the highest inhibition as compared to STD.UIF. The significance of these inhibitions or stimulations produced by the respective UIFS in terms of their cultures mean cpm, relative to the

mean cpm of STD.UIF cultures are described in a T-test comparison, (Table 2.2.J).

Thus the effects of the respective UIFS were all significantly different from STD.UIF.

The relative mean percent inhibition of the respective UIFS in terms of STD.UIF (20 and 40 uls) with 10% FCS were as follows: At 20 ul UIF, all UIFS were stimulatory (i.e. produced relatively less inhibition) with respect to STD.UIF, except B.UIF which was inhibitory. At 40 ul UIF, AD, NAD and B UIFS were more inhibitory than STD.UIF, while φfree, 5M, DZ and F.TH UIFS were stimulatory.

The significance of these inhibitions or stimulations by the respective UIFS in terms of their cultures mean cpm, relative to the mean cpm STD.UIF cultures are described below in a t.test comparison, (Table 2.2.K)

Thus at 20 ul, AD, NAD, DZ and F.TH UIFS were not significantly different from STD.UIF while φfree, 5M and B UIFS were different. At 40 ul all UIFS were significantly different from STD.UIF.

Comparing the mean percent inhibitions or stimulations produced by the respective UIFS (20 and 40 uls) between cells cultured with and without 10% FCS have shown that at 20 ul UIF: STD, φfree, 5M, DZ and F.TH UIFS were more inhibitory with 10% FCS than without FCS. AD, NAD and B UIFS were relatively less inhibitory with 10% FCS than without FCS. At 40 ul UIF: STD, φfree, 5M, DZ and F.TH UIFS were more inhibitory with 10% FCS than without FCS, while AD, NAD and B UIFS were relatively less inhibitory with 10% FCS than without FCS.

The significance of the difference between the percent inhibitions or stimulations between cells cultured with or without 10% FCS in the presence of 20 ul and 40 ul of UIF, are described below in a t-test comparison, (Table 2.2.L)

Thus at 20 ul, all UIFS were significantly different, except for ϕfree and 5M UIFS. At 40 ul, all UIFS were significantly different, except for STD and ϕfree which were not different.

Table 2.2.A4: A reference table for tables 2.2.A1, A2 and A3, for mean percent inhibitions produced by the respective UIFS on cells cultured with and without 10% FCS at 0/4, 2/4 and 4/4 incubation periods.

	Cultured without 10% FCS			Cultured with 10% FCS		
UIF Type	0/4	2/4	4/4	0/4	2/4	4/4
20 ul						
STD	14.8	15.03	11.34	5.17	18.82	28.29
AD	29.8	30.03	33.77	32.4	35.89	23.33
NAD	30.8	28.72	31.30	41.6	36.31	23.59
ϕ Free	0.09	0.27	+4.23	+5.62	+0.53	9.77
5M	0.67	1.29	+6.06	+11.76	+1.56	4.14
DZ	0.5	+2.52	+3.53	+12	+6.76	18.20
FTH	6.1	5.28	+11.34	0.7	16.45	23.82
B	66.8	71.07	76.18	63.8	66.04	68.4
40 ul						
STD	31.6	32.18	33.75	19.89	21.32	37.79
AD	46.1	49.65	59.48	43.44	48.86	46.33
NAD	50.2	51.22	57.51	37.62	45.09	43.36
ϕ Free	4.67	6.28	1.16	4.75	+3.06	7.95
5M	11.8	9.06	5.07	+4.4	3.66	14.58
DZ	1.4	+1.39	+2.68	+4.6	+1.76	21.60
FTH	12.5	17.72	16.16	12.9	19.95	31.77
B	83.4	85.44	89.14	75.6	78.89	82.23

+ = stimulation of uptake.

STD UIF = Standard UIF(spleen white cell fraction without red cells);
AD UIF = Adherent cells (of the white cell fraction spleen);
NAD UIF = Non-adherent cells (of white cell fraction spleen);
ϕ Free UIF = White cell fraction of the spleen without macrophages;
5M UIF = STD UIF, but cultured at half original density of 5×10^7 cells per petr dish.
DZ UIF = dialysed STD UIF; FTH UIF = Freeze thawed (white cell fraction spleen); BUIF = BC_3H-1 muscle cell line UIF.

Results:

The mean cpm for mouse thymocyte cultures cultured without FCS was 557 cpm = (medium controls) and for cultures cultured with 10% FCS was 287 cpm = (10% FCS Controls) as described in Table 2.2.81, incubation sequence 0/4 . There was an inhibition of 56.33% due to FCS, which was significant, ($t=5.56$, $P<.01$).

For cells cultured without FCS, the highest mean percent inhibition of isotope uptake by cell cultures in the UIFS of the spleen group, was by STD.UIF at 59%, although AD and NAD UIFS were in the same region of isotope inhibition uptake at around 56 to 52%. B.UIF produced less inhibition and was around 42%, followed by DZ at 32% while the least inhibitory was φfree.UIF at 13%. SM.UIF was fairly stimulatory with 20% more uptake, but F.TH.UIF was most stimulatory, achieving +80% uptake of 125 IUDR by thymocyte cultures, which was not the case with plasmcytoma cultures. At 40 ul UIF there was some increase in the inhibition of isotope uptake by cell cultures, although not a substantial one for all the UIFS. Again, STD, AD and NAD UIFS produced the highest inhibition in the spleen group and were in the region of 61 to 68% inhibition, followed by B.UIF at around 57% inhibition. DZ produced 41% inhibition while φfree was slightly weaker at around 28% inhibition.

The significance of the stimulations or inhibitions produced by the respective UIFS (20 and 40 ul per well) in terms of their cultures mean cpm, relative to the mean cpm of control cultures, are described in a T-test comparison, (Table 2.2.M).

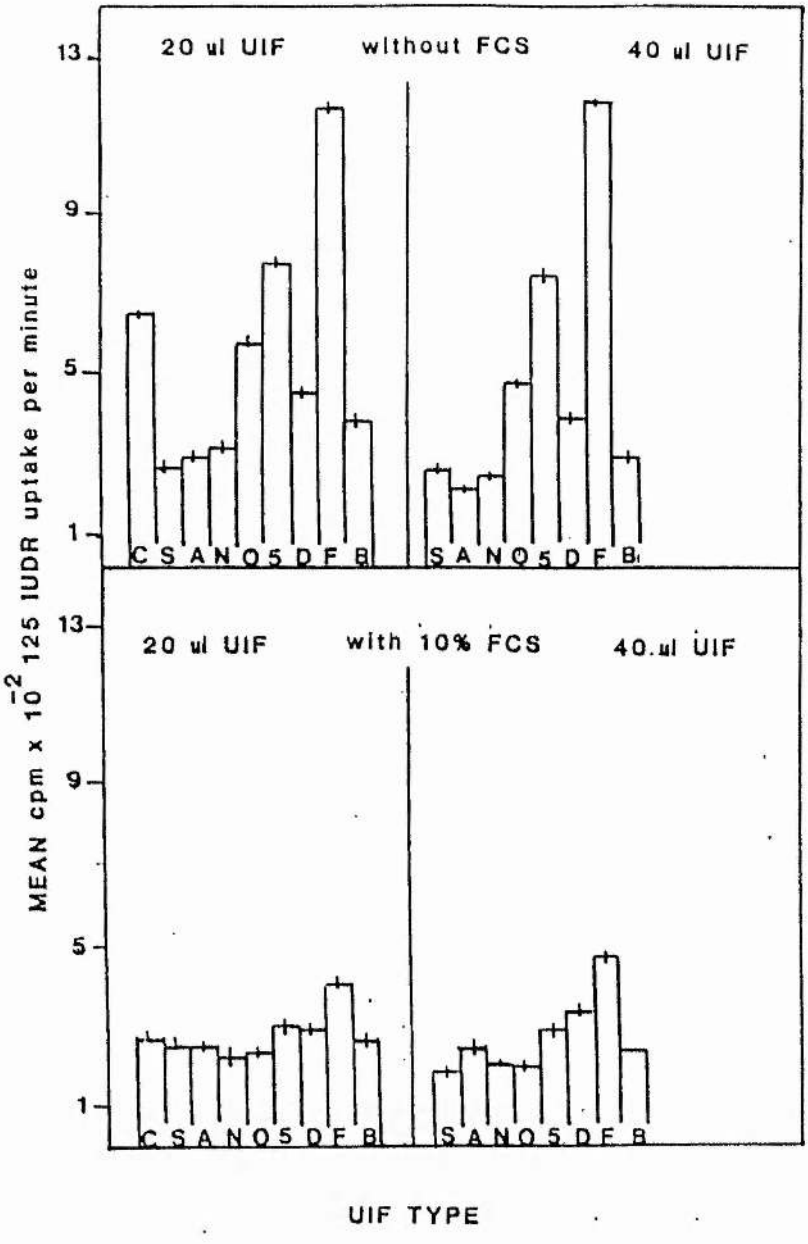


FIGURE 2.2.B1: The uptake of 125IUDR by mouse thymocyte cultures, cultured without and with FCS-HI, for 4 hours with rat UIF. 0/4 incubation sequence. C: control cultures without UIF. S: standard UIF. A: adherent cells UIF. N: non-adherent cells UIF. O: macrophage free UIF. 5: standard UIF, produced at half its culture density. D: dialysed UIF. F: freeze thaw UIF. B: muscle cell UIF.

Table 2.2.B1: The effect of several types of Rat UIF on the uptake of 125IUDR by mouse thymocyte cultures, cultured with or without 10% FCS for a 0/4 hour incubation period. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± S.E. is included.

	Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
STD 20 ul	265 ± 35	59.66 ± 5.4	266 ± 23	7.3 ± 8.1
AD	283 ± 18	56.86 ± 2.8	262 ± 21	8.71 ± 6.8
NAD	315 ± 21	52 ± 3.3	235 ± 29	17.93 ± 10.2
∅ Free	570 ± 38	13.18 ± 5.8	244 ± 21	14.94 ± 7.3
5M	791 ± 17	+20.38 ± 2.5	309 ± 27	+7.69 ± 9.7
DZ	448 ± 25	31.83 ± 3.8	306 ± 20	+6.65 ± 7.1
FTH	1183 ± 71	+80.01 ± 10.9	417 ± 40	+45.31 ± 14.1
B	380 ± 23	42.2 ± 3.5	273 ± 7	4.82 ± 2.4
STD 40 ul	255 ± 24	61.18 ± 3.6	199 ± 12	30.66 ± 4.4
AD	205 ± 26	68.76 ± 3.9	249 ± 22	13.20 ± 7.8
NAD	235 ± 18	64.22 ± 2.8	215 ± 29	24.97 ± 10.1
∅ Free	471 ± 50	28.27 ± 7.7	211 ± 18	26.22 ± 6.5
5M	747 ± 34	+13.72 ± 4.6	304 ± 35	+6.16 ± 12.2
DZ	384 ± 25	41.52 ± 3.4	344 ± 16	+20.09 ± 5.6
FTH	1197 ± 79	+82.14 ± 12.1	474 ± 29	+65.09 ± 10.3
B	283 ± 37	56.89 ± 5.6	243 ± 16	15.33 ± 5.8
Control	657 ± 70	0.0	287 ± 18	0.0

+ = Stimulation of uptake; STD = StandardUIF; AD = Adherent cells of STD UIF; NAD = Non-adherent cells of STD UIF; ∅ Free = Macrophage free STD UIF; 5M = STD UIF at half culture density; DZ = Dialysed STD UIF; FTH = Freeze thawed STD UIF; B = BC_3H-1 muscle cell line UIF.

Thus at 20 and 40 ul all comparisons were significant except for φfree and 5M which were not.

For cells cultured with 10% FCS, with 20 ul of UIF, the highest inhibition of isotope uptake in the spleen group was by NAD UIF at 17.9% followed by a nearly similar inhibition by φfree UIF at around 15%. STD and AD UIFS were inhibitory, around a similar amount of 7-8%, while the least inhibitory was B.UIF at 4.8%. 5M and DZ UIFS were stimulatory at about the same amount of 6-7% while F.TH UIF was approximately 6 times more stimulatory than 5M UIF producing an increase of 45% 125 IUDR uptake. At 40 ul UIF, there was a fair increase in the effects on isotope uptake by cell cultures, whether inhibitory or stimulatory except for 5M UIF which had a similar value to that at 20 ul per well.

The significance of the stimulations or inhibitions of the respective UIFS in terms of their cultures mean cpm, relative to the mean cpm of the control cultures has been described in a T-test comparison, (Table 2.2.N).

Thus at 20 ul UIF, the mean cpm of cultures in all UIFS were not different from the mean cpm of culture control values except for F.TH. At 40 ul, AD, NAD, 5M and B UIFS were not significantly different from controls, while STD, φfree, DZ and F.TH were different.

The relative inhibitory or stimulatory effects of the respective UIFS in terms of STD.UIF for cells cultured without FCS are as follows: At 20 ul UIF all UIFS were stimulatory, i.e. (less inhibitory than STD.UIF), and at 40 ul all UIFS were stimulatory except for AD and NAD UIFS which were slightly more inhibitory. The significance for the respective stimulations or inhibitions of the

respective UIFS in terms of their cultures mean cpm, relative to the mean cpm of STD.UIF cultures are described below in a T-test comparison, (Table 2.2.0)

Thus at 20 ul UIF, the mean cpm of cell cultures, cultured in all UIFS were different from the mean cpm of STD.UIF cultures except for AD and NAD UIFS which were not. At 40 ul UIF, the mean cpm of cultures, cultured in all UIFS were different from the mean cpm STD.UIF culture except for AD, NAD and B UIFS.

The relative inhibitory or stimulatory effects of the respective UIFS in terms of STD.UIF for cells cultured with 10% FCS are as follows: At 20 ul UIF, all UIFS were relatively stimulatory (i.e. less inhibitory than STD.UIF) in terms of STD.UIF, except AD, NAD and φfree UIFS which were inhibitory. At 40 ul, all UIFS were stimulatory (less inhibitory) in terms of STD.UIF. The significance of the relative stimulations or inhibitions by the respective UIFS in terms of their cultures mean cpm, relative to the mean cpm of STD.UIF cultures are described in at T-test comparison, (Table 2.2.P).

Thus at 20 ul UIF per well, the mean cpm of cultures, cultured in all UIFS were not significantly different from the mean cpm of STD.UIF cultures except for F.TH.UIF. At 40 ul UIF, the mean cpm of cultures in all UIFS were not different from the mean cpm of STD.UIF cultures except for 5M, DZ and F.TH UIFS which were significantly different.

Comparing the percent inhibitions or stimulations between cells cultured with and without 10% FCS at 20 ul UIF, have shown that all the UIFS whether stimulatory or inhibitory, except φfree.UIF, produced less inhibition or stimulation when cultured with 10% FCS, than without FCS. φfree.UIF produced a slight increase in inhibition with

10% FCS, compared to non FCS cultures. At 40 ul, all UIFS produced less inhibitions or stimulations with 10% FCS, as compared to FCS lacking cultures. The significance of the difference between the percent inhibitions or stimulations between cells cultured with or without 10% FCS in the presence of 20 ul or 40 ul of UIF, is described in a T-test comparison, (Table 2.2.Q)

Thus at 20 or 40 ul UIF per well all differences were significant except for ϕfree, 5M and F.TH UIFS which were not different.

Table 2.2.H

UIF cpm VS Control cpm (without FCS)

	20 ul	40 ul
STD:	P*/*	P**
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P*	P*
5M:	P*	P*
DZ:	P*	P*
FTH:	P*/*	P**
B:	P**	P**

Table 2.2.I

UIF cpm VS Control cpm (10% FCS)

	20 ul	40 ul
STD:	P**	P**
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P*	P*
5M:	P*	P**
DZ:	P**	P**
FTH:	P**	P**
B:	P**	P**

Table 2.2.J

UIF type VS STD UIF (without FCS)

	20 ul	40 ul
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P**	P**
5M:	P**	P**
DZ:	P**	P**
FTH:	P**	P**
B:	P**	P**

Table 2.2.K

UIF type VS STD UIF (10% FCS)

	20 ul	40 ul
AD:	P*	P**
NAD:	P*	P**
ϕFr:	P**	P**
5M:	P**	P**
DZ:	P*	P**
FTH:	P*	P*/*
B:	P**	P**

Table 2.2.L

UIF.FCS VS UIF (without FCS)

	20 ul	4o ul
STD:	P**	P*
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P*	P*
5M:	P*	P**
DZ:	P**	P**
FTH:	P**	P**
B:	P*/*	P**

Table 2.2.M

UIF cpm VS Control cpm (without FCS)

	20 ul	40 ul
STD:	P**	P**
AD:	P**	P**
NAD:	P**	P**
ϕFr:	P*	P*
5M:	P*	P*
DZ:	P*/*	P**
FTH:	P**	P**
B:	P**	P**

Table 2.2.N

UIF cpm VS Control cpm (10%FCS)

	20 ul	40 ul
STD:	P*	P**
AD:	P*	P*
NAD:	P*	P*
ϕFr:	P*	P*/*
5M:	P*	P*
DZ:	P*	P*/*
FTH:	P*/*	P**
B:	P*	P*

Table 2.2.O

UIF type VS UIF STD (without FCS)

	20 ul	40 ul
AD:	P*	P*
NAD:	P*	P*
ϕFr:	P**	P**
5M:	P**	P**
DZ:	P**	P**
FTH:	P**	P**
B:	P*/*	P*

Table 2.2.P

UIF type VS UIF STD (10% FCS)

	20 ul	40 ul
AD:	P*	P*
NAD:	P*	P*
ϕFr:	P*	P*
5M:	P*	P*/*
DZ:	P*	P**
FTH:	P*/*	P**
B:	P*	P*

Table 2.2.Q

UIF FCS VS UIF (without FCS)

	20 ul	40 ul
STD:	P**	P**
AD:	P**	P**
NAD:	P*/*	P**
ϕFr:	P*	P*
5M:	P*	P*
DZ:	P**	P**
FTH:	P*	P*
B:	P**	P**

Results:

The mean cpm for mouse thymocyte cultures, cultured without FCS was 145 cpm (medium controls) and for cells cultured with 10% FCS was 226 cpm (10% FCS controls) as described in Table 2.2.B2, incubation sequence 2/4 . There was a stimulation of 55.17% due to FCS, which was not significant, ($t=2.162$, $P>.05$).

The mean percent of isotope uptake by cell cultures (without FCS), with 20 and 40 ul UIF were either stimulatory or inhibitory. However the mean cpm of cell cultures in the respective UIFS were all near culture control mean cpm value, except for the mean cpm of cultures, cultured with F.TH and 5M UIFS which were relatively higher.

The significance of these inhibitions or stimulations produced by the respective UIFS in terms of their cultures mean cpm, relative to the mean cpm of culture controls, are described below in a t-test comparison, (Table 2.2.R).

Thus at 20 ul UIF per well, the mean cpm of cell cultures in all UIFS were not different from the mean cpm of culture controls except for the mean cpm of cultures, cultured with F.TH UIF which significantly different. Similarly at 40 ul, the mean cpm of cultures in all UIFS were not different except for the mean cpm of cultures in 5M and F.TH UIFS which were significantly different, from the mean cpm of culture controls.

The mean percent of isotope uptake by cells cultured with 10% FCS, and 20 or 40 ul UIF, were either inhibitory or stimulatory in their effects on the uptake of 125 IUDR by thymocytes. However, as

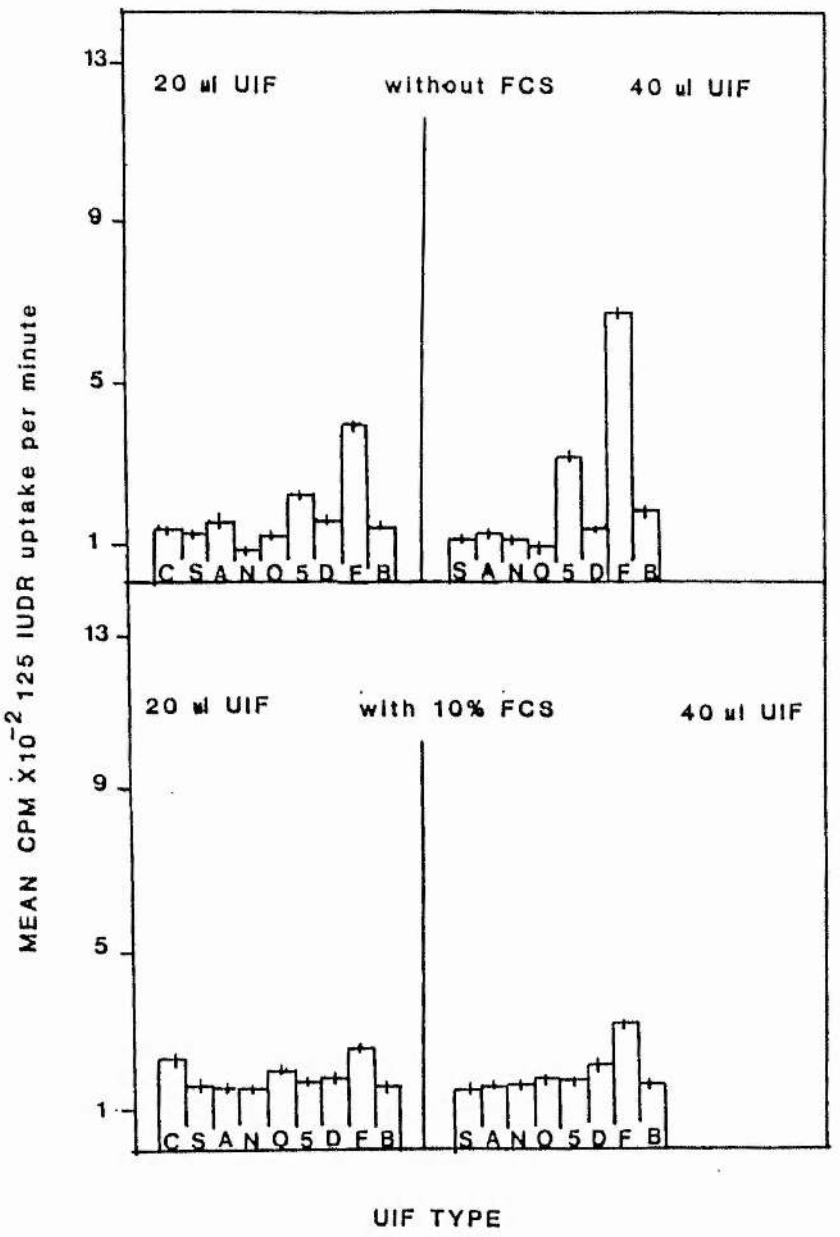


FIGURE 2.2.B2: The uptake of 125IUDR by mouse thymocyte cultures, cultured without and with FCS-HI, for 4 hours with rat UIF. 2/4 incubation sequence. The UIF types are described in Fig. 2.2.B1.

Table 2.2.B2: The effects of Rat UIF on the uptake of 125IUDR by mouse thymocyte cultures, cultured with or without 10% FCS for a 2/4 hour incubation period. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± S.E. is included.

	Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
STD 20 ul	130 ± 29	10.34 ± 20.1	172 ± 28	23.89 ± 12.4
AD	156 ± 25	7.58 ± 15.9	176 ± 16	22.12 ± 7.1
NAD	88 ± 4	39.31 ± 2.7	176 ± 18	22.12 ± 8
ø Free	127 ± 13	12.46 ± 9	207 ± 11	8.13 ± 5.1
5M	229 ± 32	+57.61 ± 20	181 ± 20	19.89 ± 9.2
DZ	164 ± 4	+12.67 ± 14	190 ± 15	15.64 ± 6.9
FTH	398 ± 39	+173.44 ± 27.4	267 ± 18	+18.12 ± 8.1
B	149 ± 17	+2.68 ± 12.1	168 ± 26	25.66 ± 10.4
STD 40 ul	112 ± 12	22.75 ± 8.2	155 ± 20	31.41 ± 8.9
AD	135 ± 26	6.89 ± 14.2	167 ± 20	26.10 ± 9.1
NAD	114 ± 10	21.38 ± 6.7	171 ± 23	24.33 ± 10.5
ø Free	97 ± 10	33.31 ± 7.4	189 ± 31	16.26 ± 1.4
5M	322 ± 19	+121.3 ± 13.5	178 ± 21	21.23 ± 9.3
DZ	149 ± 9	+2.75 ± 6	219 ± 13	3.09 ± 5.9
FTH	682 ± 64	+367.99 ± 44.3	331 ± 39	+46.46 ± 17.5
B	188 ± 23	+29.03 ± 16.4	174 ± 22	23.07 ± 7.6
Controls	145 ± 28	0.0	226 ± 23	0.0

+ = Stimulation of uptake; STD = Standard UIF; AD = Adherent cells of STD UIF; NAD = Non-adherent cells of STD UIF; ø Free = Macrophage Free STD UIF; 5M = STD UIF at half culture density; DZ = dialysed STD UIF; FTH = Freeze thawed STD UIF; B = BC_3H-1 muscle cell line UIF.

was the case with the FCS lacking cultures, the mean cpm of cultures in the respective UIFS were all near the mean cpm value of control culture, except for the mean cpm of cultures, cultured in 40 ul F.TH UIF which was relatively higher. However the mean cpm of cell cultures, cultured in all the respective UIFs were not significantly different from the mean cpm of control cultures as described in the t-test comparison below. The significance of the respective inhibitions or stimulations produced by the respective UIFS in terms of their cultures mean cpm, relative to the mean cpm of control cultures, are described in a t-test comparison, (Table 2.2.S).

Comparing the mean percent inhibitions or stimulations produced by the respective UIFS (20 and 40 uls) between cells cultured with and without 10% FCS have shown that STD, AD, (NAD, 40 ul), SM, DZ, F.TH and B UIFS were all more inhibitory with 10% FCS than their counterparts without FCS, while NAD.(20 ul) and Øfree UIFS were less inhibitory with 10% FCs than without FCS. The significance of the difference between the percent inhibitions or stimulations between cells cultured without or with 10% FCS in the presence of 20 and 40 uls of UIF is described in a t-test comparison, (Table 2.2.T).

Thus at 20 ul UIF all comparisons were not significant except for SM, DZ and F.TH UIFS. At 40 ul UIF all comparisons were not significant except for SM, F.TH and B UIFS which were significantly different.

Results:

The mean cpm for mouse thymocyte cultures cultured without FCS was 145 cpm (medium control), and for cells cultured with 10% FCS was 126 cpm (10% FCS control), as described in Table 2.2.B3, incubation sequence 4/4 . There was an inhibition of 13.10% due to FCS

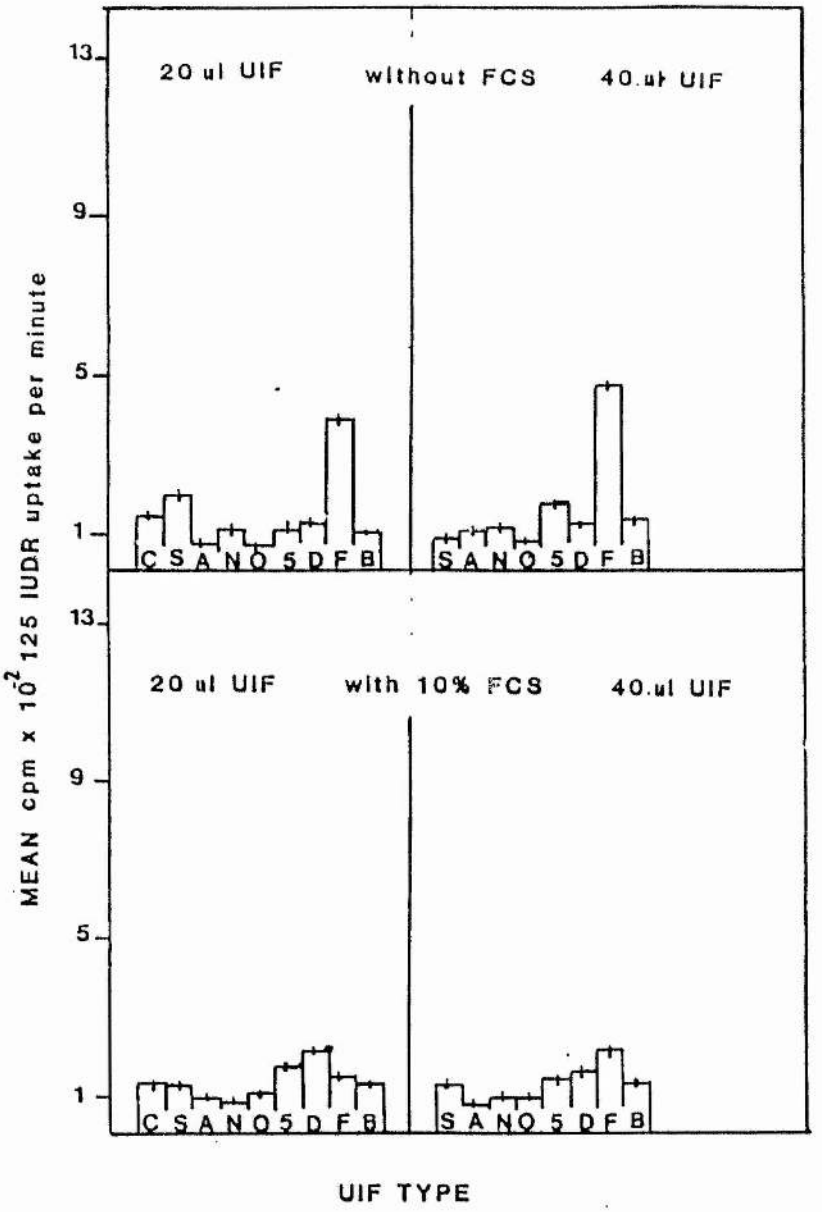


FIGURE 2.2.B3: The uptake of 125IUDR by mouse thymocyte cultures, cultured without and with FCS-HI, for 4 hours with rat UIF. 4/4 incubation sequence. The UIF types are described in Fig.2.2.B1.

Table 2.2.B3: The effects of Rat UIF on the uptake of 125IUDR by mouse thymocyte cultures, cultured with or without 10% FCS for a 4/4 hour incubation period. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± S.E. is included.

	Cultured without FCS		Cultured with 10% FCS	
UIF type and concentration per well	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
20 ul				
STD	196 ± 15	+35.17 ± 10.9	124 ± 9	1.58 ± 7.2
AD	69 ± 7	52.48 ± 5.1	91 ± 16	27.77 ± 9.8
NAD	99 ± 9	32.07 ± 4.8	77 ± 10	38.35 ± 8.3
ø Free	65 ± 10	55.26 ± 26.8	107 ± 10	14.73 ± 8.1
5M	101 ± 22	30.34 ± 15.4	178 ± 26	+41.04 ± 21.1
FTH	386 ± 52	+166.2 ± 35.7	146 ± 9	+15.95 ± 5.9
B	99 ± 3	31.90 ± 2.3	134 ± 18	+6.33 ± 14.4
40 ul				
STD	83 ± 6	42.75 ± 4	123 ± 18	2.53 ± 14.6
AD	93.± 6	35.86 ± 3.7	74 ± 9	41.26 ± 7
NAD	111 ± 15	23.43 ± 10.9	94 ± 29	25.39 ± 21
ø Free	76 ± 12	47.44 ± 9.6	91 ± 13	27.77 ± 10.4
5M	167 ± 31	+14.99 ± 21.6	137 ± 13	+8.71 ± 10.3
DZ	121 ± 13	16.98 ± 9.5	158 ± 19	+25.39 ± 13.7
FTH	469 ± 59	+233.44 ± 40.5	207 ± 11	+64.28 ± 7
B	131 ± 17	9.65 ± 12.2	124 ± 23	1.58 ± 16.3
Control	145 ± 19	0.0	126 ± 20	0.0

+ = Stimulation of isotope uptake. STD = Standard UIF; AD = Adherent cells of STD UIF; NAD = Non-adherent cells of STD UIF; ø Free = Macrophage Free STD UIF; 5M = STD UIF at half culture density; DZ = dialysed STD UIF; FTH = Freeze thawed STD UIF; B = BC_3H-1 muscle cell line UIF.

which was not significant, (t=0.703, P>.05 NS). For cells cultured without FCS, with 20 ul UIF or 40 ul UIF the inhibition or stimulation of isotope uptake was not predictable as with previous cultures with UIF. Thus STO at 20 ul was stimulatory and at 40 ul was inhibitory. AD, NAD, ϕfree and B UIFS produced substantial inhibitions at 20 ul, whereas at 40 ul, they were less inhibitory. Similarly, 5M.UIF was inhibitory at 20 ul, while at 40 ul, it was stimulatory. DZ.UIF was nearly similar in its inhibition of isotope uptake at both UIF concentrations, while the predictable F.TH.UIF maintained its stimulation of uptake, more so at 40 ul than at 20 ul UIF per well. The significance of the mean cpm of cultures in the respective UIF in terms of the mean cpm of control culture is described in a t-test comparison,(Table 2.2.U).

Thus at 20 ul UIF, the mean cpm of cultures in all UIFS were significantly different from the mean cpm of control cultures except for STO, 5M and DZ UIFS. At 40 ul, the mean cpm of all cultures in UIF were significantly different from control cultures except for NAD, 5M, DZ and B UIFS, which were not different.

For cells cultured with 10% FCS, 20 ul UIF produced inhibitions of isotope uptake by STO, AD, NAD and ϕfree UIFS. These inhibitions increased with 40 ul UIF except for NAD which decreased. 5M, DZ, F.TH and B UIFS were stimulatory at 20 ul and at 40 ul the stimulations decreased except for F.TH which increased. However, the mean cpm of most cultures in the respective UIFS at 20 or 40 ul were not significantly different from the mean cpm of control culture as described in the t-test comparison,(Table 2.2.V).

Thus at 20 ul UIF, the mean cpm of cultures in all UIFs were not significantly different from the mean cpm value of culture control,

except for DZ.UIF which was different from controls. At 40 ul, the mean cpm of cultures in all UIFS were not significantly different from the mean cpm of culture control, except for cultures in AD and F.TH which were.

Comparing the mean percent inhibitions or stimulatins produced by the respective UIFS (20 and 40 uls) between cells cultured with and without 10% FCS have shown that at 20 ul UIF, AD, ϕfree, 5M, DZ, F.TH and B were all less inhibitory with 10% FCS than without 10% FCS, while STD and NAD.UIFS was more inhibitory with FCS than without FCS. At 40 ul UIF, STD, ϕfree, DZ, and B UIFS were all less inhibitory with 10% FCS than without FCS, while AD, 5M and F.TH UIFS were more inhibitory with 10% FCS than without FCS. The significance of the difference between the percent inhibitions or stimulations between cells cultured with or without 10% FCS in the presence of 20 ul or 40 ul of UIF, is described below in a t-test comparison, (Table 2.2. W). Thus at 20 ul UIF all comparisons were different except for NAD UIF which was not. At 40 ul all comparisons were not significantly different except for STD, DZ and F.TH UIFS which were different.

Table 2.2.R

UIF cpm VS Control cpm (without FCS)

	20 ul	40 ul
STD:	P*	P*
AD:	P*	P*
NAD:	P*	P*
ϕFr:	P*	P*
5M:	P*	P**
DZ:	P*	P*
FTH:	P**	P**
B:	P*	P*

Table 2.2.S

UIF cpm VS Control cpm (10% FCS)

	20 ul	40 ul
STD:	P*	P-
AD:	P*	P-
NAD:	P*	P*
ϕFr:	P*	P*
5M:	P*	P**
DZ:	P*	P-
FTH:	P*	P**
B:	P*	P-

Table 2.2.T

UIF FCS VS UIF without FCS

	20 ul	40 ul
STD:	P*	P*
AD:	P*	P*
NAD:	P*	P*
ϕFr:	P*	P*
5M:	P**	P**
DZ:	P*/*	P*
FTH:	P**	P**
B:	P*	P*/*

Table 2.2.U

UIF cpm VS Control cpm (without FCS)

	20 ul	40 ul
STD:	P*	P*/*
AD:	P**	P*/*
NAD:	P*/*	P*
ϕFr:	P*	P*/*
5M:	P*	P*
DZ:	P*	P*
FTH:	P**	P**
B:	P*/*	P*

Table 2.2.V

UIF cpm VS Control cpm (10% FCS)

	20 ul	40 ul
STD:	P*	P*
AD:	P*	P*/*
NAD:	P*	P*
ϕFr:	P*	P*
5M:	P*	P*
DZ:	P**	P*
FTH:	P*	P**
B:	P*	P*

Table 2.2.W

UIF FCS VS UIF (without FCS)

	20 ul	40 ul
STD:	P*/*	P**
AD:	P*/*	P*
NAD:	P*	P*
ϕFr:	P*/*	P*
5M:	P*/*	P*
DZ:	P**	P*/*
FTH:	P**	P**
B:	P*/*	P*

P*/* = .05 > P > .01
P** = <.01
P* = >.05

As described above, (section 3.2.2, Figure/Tables 2.2.A1, A2 and A3) the effects of various sources of spleen UIF on the uptake of radioisotope by cell cultures was assessed in cultures, cultured with and without FCS, at three incubation sequences 0/4, 2/4 and 4/4.

It was demonstrated (20ul UIF/without FCS) that when spleen UIF was prepared from cultures depleted of macrophages, the inhibition of radioisotope uptake was eliminated. Similarly it was demonstrated that an inhibitory factor present in STD UIF was dialyzable, as the inhibition of radioisotope uptake was also eliminated. It was also shown that the inhibitory strength of STD UIF depended on the culture density of cell cultures producing the UIF, as STD UIF produced from cells cultured at half the culture density that produced STD UIF, was not inhibitory. The inhibition of radioisotope uptake at 40ul was generally higher than counterparts at 20ul. Adherent and non-adherent UIF was (surprisingly) more inhibitory than STD UIF at both UIF concentrations.

The effects of FCS on the inhibitory potential of UIF were varied. For STD UIF, there was a marked decrease in inhibition relative to counterpart cultures (without FCS), however, the inhibition of uptake increased (with FCS) with respect to the incubation sequence which was not evident in counterpart cultures. The inhibition of radioisotope uptake by adherent and non adherent UIFS was slightly lower at the 4/4 sequence than counterpart cultures, (without FCS). For dialyzed and freeze thaw (FTH) UIFS, there was a marked inhibition of radioisotope uptake at 4/4 and 2/4, 4/4 sequences respectively relative to counterparts (without FCS).

5M and macrophage free UIFS were rather similar in both environments (with and without FCS), however, at 40ul of UIF, 5M and macrophage free were more inhibitory at the 4/4 sequence than counterpart cultures (without FCS). At 40ul, the inhibition produced by STD and AD UIFS were similar in both environments, while that of NAD was reduced with FCS relative to conterparts without FCS. The inhibition of radioisotope uptake by dialyzed and FTH UIFS in both environments were similar to that of that of the 20ul group. Thus the inhibitory effect of spleen UIF depended on several factors such as , the culture density of UIF producing cultures, and the presence or absence of macrophages in culture preparations of UIF producing cultures.

The effect of the respective spleen UIFS on the uptake of radioisotope by mouse thymocytes, (Figures/Tables 2.2.B1, B2 and B3) were also variable with respect to the incubation sequence and the presence or absence of FCS.

At the 0/4 incubation sequence (20ul without FCS), the effects on radioisotope uptake by thymocytes were relatively greater than counterpart plasmacytoma cultures. The most noteworthy feature was the stimulating effect of FTH UIF on the uptake of radioisotope which persisted with the thymocyte cultures in all culture environments and incubation sequences. At 20ul (UIF-FCS) all the inhibitory effects of UIF were eliminated as compared to counterpart cultures without FCS. However, at 40ul, the effects of the respective UIFS and FCS were different. The inhibitiory effect of STD UIF was reduced substantially, that of macrophage free was maintained at the same level of inhibition while that of AD and NAD UIFS were reduced to a level of insignificance relative to counterparts cultured without FCS.

The change in uptake at 2/4 and 4/4 sequences was not as definitive as that of the plasmacytoma cultures, (where there was some increase associated with an increase in incubation sequence) as the change of radioisotope uptake by the respective UIFS was not consistent with an increase in the incubation sequence. The uptake of FCS by control cultures was relatively quite low, so was the uptake of most of the respective UIF cultures, that the relevance in the change of radioisotope uptake may be doubtful. However, the change of radioisotope uptake by cell cultures incubated with FTH UIF was consistent, in the sense that the stimulation of uptake persisted in the respective incubation sequences, although the uptake was relatively less with, than without FCS, but remained significantly different from the uptake of control cultures.

Experimental procedures:

Cell suspensions of plasmacytoma or mouse thymocytes were prepared in RPMI medium (with or without 10% FCS) and were dispensed (each type of cell suspension) into sterile tubes for lymphocyte culture (NUNC-UK) at 1.5 x 10^6 cells/ml. Each cell suspension was cultured in the presence of 20% UIF except for control cell cultures which were not. The tubes were capped and were incubated for 4, 8 or 12 hours in a Leec incubator at 37°C. Each type of culture was replicated three times. Following the incubation, aliquots from each tube were assessed for cell viability and cell number using the trypan blue exclusion test in an improved neubauer haemocytometer (Hawksley Ltd UK). Group A was cultured with 10% FCS, Group B was cultured without FCS.

Results:

The values described in Table 2.2.C0 were calculated as the difference in cell numbers between control culture mean cell number and the mean cell number in the respective UIF culture, which was related to control's mean cell number.

The mean viable cell numbers of plasmacytoma or thymocytes were either slightly above (+) or below (−) the 100% control values of culture controls, and all were not significantly different from the mean number of control cultures ($P>.05$).

Table 2.2.C0: The cell viabilities of plasmacytomas and thymocytes cultured in UIF.

	Percent change in cell number ± S.E.								
UIF Type	Plasmacytoma cells Time (hours) 4	8	12	P	c	Mouse thymocytes Time (hours) 4	8	12	P
TC [1]	-1.8 ± 1.28			*	100	+0.57 ± 5.3			*
PWC	-1.65± 0.88			*	100	-0.09 ± 0.14			*
WCt	-2.53± 7.62	-3.52 ± 1.16	+3.03 ± 10.78	* * *	100	+8.12 ± 7.46	-0.11 ± 0.11	-10.38 ± 7.61	* * *
LN	+2.41± 2.35			*	100	-0.95 ± 0.47			*
PM	-3.45 ± 2.35			*	100	-0.68 ± 0.29			*
AD			+14.54 ± 5.56	*	100				
NAD			+ 4.84 ± 2.64	*	100				
56°			+ 4.24 ± 7.15	*	100				
B			- 1.43 ± 5.91	*	100				
Wcf [2]	+11.73	+11.73 ± 2.81		*/*	100		-1.05 ± 5.9		*
AD	+ 5.15	+ 5.15 ± 2.57		*	100		-6.65 ± 1.25		*
NAD	+ 4.22	+ 4.22 ± 1.87		*	100		-6.70 ± 3.25		*
B	- 5.01	- 5.01 ± 1.70		*	100		-7.69 ± 2.82		*

P = t-test value.
c = control cultures. (100% without change)

1 with FCS $P^{*/*} = .05 > P > .01$
2 without FCS $P^{*} = > .05$

3.2.3: UIF Reversibility: the effects of exposing cell cultures to UIF for a short a period, on the uptake of 125 IUDR by cell cultures without the presence of UIF in the same cultures.

Experimental procedures:

A suspension of plasmacytoma cells was prepared and divided into 2 groups, the first contained 10% FCS in RPMI, the other was without FCS. Five replicate sterile tubes for lymphocyte culture (NUNC.UK) received 1 ml of plasmacytoma cells (1.25×10^5 cells/ml) without FCS, each. Another five replicate tubes received 1 ml each of cell suspension containing 10% FCS. Similarly another set of tubes received 1 ml each of plasmacytoma cells that contained 20% UIF STD; (5 tubes with 10% FCS and 5 without FCS).

All the tubes were incubated for 4 hours at 37°C, after which they were all centrifuged at 1.4×10^3 rpm for 5 minutes and the supernate discarded. The tubes that contained 10% FCS were resuspended in 1 ml of prewarmed RPMI-10% FCS, the other 10 tubes were resuspended in RPMI without FCS. Each tube received 100 ul of 125 IUDR (1 uci per culture) and were incubated for 4 hours. The procedures for preparing rat STD-UIF and the assessment of IUDR uptake by cell culture were described earlier.

Results:

The uptake of 125 IUDR was nearly similar by UIF cell cultures and control cultures as described in Table 2.3 , as there was a slight decrease in the mean Cpm of both UIF test cultures, compared to the mean Cpm of their respective control cultures. The difference in the mean Cpm between UIF cultures and controls (without FCS) was not significant, ($t=0.874$, $P>.05$), so was the difference between the mean Cpm of UIF cultures and controls (with 10% FCS) which was not, ($P>.05$)

Table 2.3: The reversible effects of Rat UIF on the uptake of 125IUDR by plasmacytoma cells. The values are expressed as mean cpm with ± S.E. values included.

	Without 10% FCS	With 10% FCS
	Mean cpm ± S.E.	Mean cpm ± S.E.
STD UIF	34284 ± 716	33658 ± 5498
Control:without UIF	34972 ± 328	31671 ± 2642

STD UIF = Standard UIF, white cell fraction of spleen.

3.2.4: The effect of STD.UIF on the passage of cells from S phase to mitosis, and the number of arrested metaphases.

Experimental procedures:

A: The optimum dosage of vincristine sulphate (Oncovin) required for arresting the maximum number of metaphases at several incubation periods. A cell suspension of plasmacytoma cells in 20% FCS was prepared, and aliquots of 0.5 mls were dispensed into 45 sterile tubes (NUNC.UK) for lymphocyte culture. The tubes were grouped into 3 groups of 15 tubes each. The tubes in the first group received an equal volume (0.5 ml) of 1 ug/ml onconvin each, the second received 2.6 ug/ml oncovin , and the third group received 8 ug/ml. Thus the final concentration of oncovin in 10% FCS in the first group was 0.5 ug/ml, in the second group: 1.3 ug/ml and in the third group: 4 ug/ml. Three tubes of each oncovin group were incubated for the following periods in hours at 37°C: 0.5, 1, 2, 3 and 4 hours i.e. 9 tubes per incubation period. After each incubation Period, Cytopsin-smears of each tube were prepared and stained in Jonner-Giemsa. The Cytopsin slides were later scored for metaphase counts at 10 × 40 magnification . At least 2000 cells per slide were screened for metaphases.

B: STD.UIF was incubated with plasmacytoma cells for 4 hours, after which, the optimum dosage of oncovin was applied to the cell culture for a specified time period. A plasmacytoma cell suspension was prepared as described previously and dispensed as 950 ul aliquots into sterile tubes for lymphocyte culture. Two sets of 5 tubes each were incubated for 4 hours at 37°C. The first set of tubes contained a suspension of plasmcytoma cells in 10% FCS and 20% of STD.UIF. The second set had a plasmocytoma cell suspension in 10% FCS without STD.UIF. Following the 4 hour incubation period, 50 ul of RPMI.10%

FCS that contained 0.5 ug of oncovin were dispensed into each tube of the two sets, and were incubated for a further 2 hour period at 37°C. Another 2 sets of 5 tubes each received 1 ml per tube of plasmacytoma suspension in 10% FCS. The first set of plasmacytoma suspension was cultured in the presence of STD.UIF at a concentration of 20%. The other set was without STD.UIF, and served as a control culture for the experiment. The 2 sets of tubes were incubated for 4 hours at 37°C. Cytopsin slides were prepared and metaphases counted as described above and 4 slides of each set were assessed for their metaphase count.

Results:

The effect of oncovin on arresting metaphases was linear in the case of the 0.5 and 1 ug groups, between 0.5 and 2 hours, and decreased thereafter between 2 to 4 hours as described in Figure/Table 2.4.B . In the 4 ug group, the number of arrested metaphases were nearly constant, between 0.5 to 3 hours, which decreased thereafter at 4 hours. The highest metaphase count of 107 was for the 0.5 ug group at 2 hours of incubation. In the 0.5 ug group, the difference in metaphase numbers between the 0.5 hour and 1 hour incubation periods was not significant, ($t=1.943$, $P>.05$), while the difference between the 0.5 hour and 2 hours incubation periods was significant, ($t=3.215$, $0.05>P>.01$). At the 2 hour incubation period, the difference between the 0.5 ug and 1.3 ug groups was not significant, ($t=1.749$, $P>.05$), while the difference between the 0.5 ug and 4 ugs groups was significant, ($t=2.941$, $0.05>P>.01$).

The mean metaphase count for plasmacytoma cultures cultured with vincristine and UIF as described in Table 2.4.B were as follows: in the PHV2 group, (plasmacytoma cultures with vincristine) the mean metaphase count was the highest at 141 metaphases, while the STD.PHV2

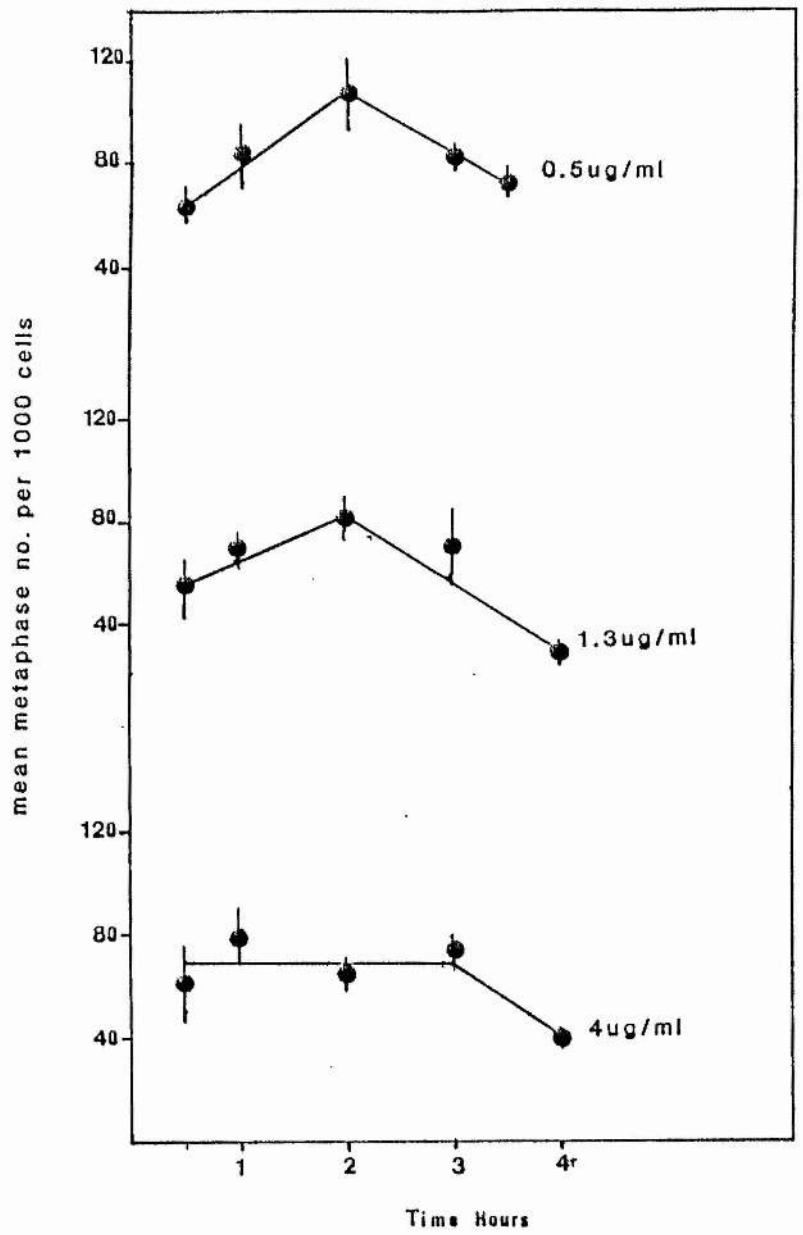


FIGURE 2.4A: The accumulation of metaphase figures in plasmacytoma cultures, cultured with different concentrations of vincristine sulphate, for different periods of incubation time.

Table 2.4.A: The effects of different concentrations of vincristine sulphate in in-vitro cultures, on the collection of metaphases at different incubation periods of time. The metaphase counts are expressed as the mean metaphase number per 1000 cells, with ± standard error values included.

	Vincristine sulphate concentration : ug/ml		
	0.5	1.5	4
Time. (hours)	(mean metaphase numbers ± S.E.)		
0.5	63 ± 5	54 ± 13	62 ± 16
1	84 ± 12	69 ± 5	81 ± 11
2	107 ± 16	82 ± 7	66 ± 5
3	83 ± 4	70 ± 16	75 ± 4
4	73 ± 6	29 ± 5	40 ± 2

Table 2.4.B: The effects of STD UIF and vincristine sulphate in in-vitro cultures on the collection of metaphases. The metaphase counts are expressed as the mean metaphrase number per 1000 cells, with ± standard error included.

Code	mean metaphase number ± S.E.
F + V2	141 ± 24
STD.F + V2	103 ± 3
STD.F	113 ± 3
F	107 ± 15

F + V2 =Plasmacytoma cultures, cultured in 10% FCS, incubated for 4 hours, followed by the addition of oncovin, followed by a further incubation of 2 hours.

STD.F + V2 = Plasmacytoma cultures cultured in 10% FCS and 20% UIF, incubated for 4 hours, then oncovin was added, and the cell cultures were incubated for a further 2 hours.

STD.F = Plasmacytoma cells in 10% FCS and 20% UIF, incubated for 4 hours.

F = Plasmacytoma cells in 10% FCS incubated for 4 hours.

group, (plasmacytoma cultures with UCF and vincristine) had a mean metaphase count of 103. The difference between the F+V2 and STD.F+V2 groups was not significant, ($t=1.498$, $P>.05$. The STD.F group, (plasmacytoma cultures with STD.UCF) had a mean metaphase count slightly higher than F group, (plasmacytoma cultures in FCS), (113.12.VS.107) which was not significant, ($t=0.373$, $P>.05$). The UCF, therefore, decreased the mitotic index slightly as in the STD.F+V2 group but was not different from the F+V2 group.

3.2.5: The effects of STD.UCP on the Labelling Index and grain count of plasmacytoma cells.

Experimental procedures:

A suspension of plasmacytoma cells was prepared as described previously, and was divided into 3 groups. The first group was a suspension in RPMI without HI-FCS and STD.UCP. The second suspension was with 10% FCS, while the third group contained 10% FCS and 20% STD.UCP. Aliquots of each suspension were dispensed into sterile lymphocyte culture tubes (NUNC-UK); 1 ml per tube and 5 tubes per group.

Each tube received 50 ul of RPMI containing 1 uci of ^{3}H-Tdr (5 ci/mmol sp.act). The cultures were incubated for 4 hours at 37°C. Following the incubation, samples from each group were transferred into a cytopsin centrifuge, and cytopsin-smears were prepared. The cytopsin-smears were processed for autoradiography according to the procedure of Kopriwa and Leblond (1962) and were stained in haematoxylin-eosin stain.

The slides were assessed for the Labelling Index at 10 × 40 mag., and for grain counts at 10 × 100 mag . For the determination of the (LI) Labelling Index, 4 cytopsin smear slides of each group were examined, and 4000 cells per slide were scored to determine the proportion of labelled to unlabelled cells per slide. For the determination of the grain count, 4 cytopsin slides of each group were examined, and 100 labelled cells per slide were scored for their grain count as described in materials and methods.

Results:

The highest Labelling Index for plasmacytoma cultures, as described in Table 2.5 , was for cells cultured without FCS, followed

Table 2.5: The labelling index and mean grain count of plasmacytoma cells, cultured without FCS, with 10% FCS and with 10% FCS and 20% UIF. The ± standard error is included.

	Number of cells containing the respective mean grain count ± S.E.		
Grain count Group	M	F	UF
A	3 ± 1	4.5 ± 1.5	13.25 ± 1.3
B	4.5 ± 1.5	8.5 ± 1.3	10.75 ± 0.47
C	92.5 ± 2	87.0 ± 2	76.00 ± 1
	Mean number of labelled cells ± S.E.		
	M	F	UF
	241 ± 49	181 ± 28	175 ± 19
	Percent Labelling index ± S.E.		
	24.1 ± 5.91	18.1 ± 2.86	17.5 ± 1.89
Group	Grain count and class interval		
A	0 ⟶ 50 lightly labelled		
B	50 ⟶ 100 medium labelled		
C	above 100 densely labelled.		

M = cells cultured without FCS
F = cells cultured with 10% FCS
UF = cells with 20% UIF - 10% FCS

by cells cultured with 10% FCS and cells cultured with 10% FCS - 20% UIF . The difference in the labelling indeces between cells cultured with 10% FCS and cells cultured without FCS was not significant, (t=1.174, P>.05), and neither was the difference between cells cultured with 10% FCS and cells cultured with 20% UIF-10% FCS, significant, (t=0.203, P>.05).

In class interval [0 - 50 grains per cell] lightly labelled group, the mean number of cells, cultured with UIF (UF) was 13 cells, which was the highest in this class interval, followed by cells cultured with FCS without UIF (F) and cells cultured without FCS and UIF (M), which were the lowest in cell number, at 4.5 to 3 cells respectively. The difference between the cells cultured with 20% UIF-10% FCS , and cells with 10% FCS was significant (t=4.811, P<.01 S), while the difference between cells cultured with 10% FCS and without FCS was not significant, t=0.965, P>.05 NS. The mean number of cells in class interval [50 - 100] was the highest for those cultured in UF at about 11 cells, followed by cells cultured in F , while the without FCS group had the lowest mean cell count of approximately 5 . The difference between the F and UF groups was not significant, (t=1.603, P>.05), and neither was the difference significant between the F and M groups, (t=1.96, P>.05).

In the densely labelled group [above 100 grains per cell] the mean number of cells that were cultured without 10% FCS had the highest count, at about 93 cells followed by cells that were cultured with 10% FCS, and the number of cells which were cultured with 20% UIF-10% FCS was the lowest, at 76 cells.

The difference between the 10% FCS-20% UIF and the 10% FCS groups was significant, (t=4.417, P<.01), while the difference between the 10% FCS and without FCS groups was not significant, (t=1.822,

P>.05). Thus for cells cultured with UCF-FCS there was a reduction of 12.6% compared to cells cultured in FCS without UCF in the densely labelled Group C .

3.2.6: The effect of STD.UIF on plasmacytoma colony formation in the spleens of mice.

Experimental procedures:

A: Eighty Balb/C mice were irradiated with (7.2 Gy) in an X-ray machine and were divided in 4 groups (20 mice/group). A plasmacytoma suspension was prepared as described previously in RPMI without FCS and was diluted to the following concentrations: 6×10^5 cells/ml, 4×10^5 cells/ml, 3×10^5 cells/ml and 2×10^5 cells/ml. Each mouse in the first group received 1.5×10^5 cells per 0.25 ml through a sterile needle (23 Gauge 1¼ Beckton Dickson - Ireland) into the tail vein from a 1 ml sterile syringe (Beckton Dickson - Ireland); the second group received 1×10^5 cells/0.25 ml per mouse; the third group received 7.5×10^4 cells/0.25 ml per mouse and the fourth group received 5×10^4 cells/0.25 ml per mouse. Nine days following the injections, the mice were sacrificed under ether anaesthesia, and spleens were assessed for their plasmacytoma colony content as described in materials and methods.

B: Eighty Balb/C mice were irradiated as described above, (A) and each group of 20 mice received 0.25 ml (10^5 cells/mouse) plasmacytoma suspension in RPMI without FCS per mouse, as follows: Group 1, mice received a plasmacytoma suspension which was incubated with 20% UIF.STD (4 hour incubation + 1 extra hour without UIF) at 37°C; Group 2 mice received a plasmacytoma suspension which was incubated with 20% UIF, (4 hour incubation, followed by 1 hour incubation with Ara/C (20 ug/ml)); Group 3 mice received a plasmacytoma suspension which was incubated for [4 hours in RPMI medium + 1 hour incubation with Ara/C, 20 ug/ml] at 37°C; Group 4 mice received a plasmacytoma suspension in RPMI, which was incubated for 4 hours, followed by 1 hour incubation without Ara/C. The

procedure for the preparation of the 4 types of plasmacytoma cultures has been described in detail in materials and methods.

Nine days following the injections, the mice were sacrificed under ether anaesthesia, and the spleens were assessed for their cell colony contents under a stereo microscope.

Results:

It is evident that there was a linear relation between the number of cells injected into mice, and the number of tumour colonies formed in their spleens as described in Table 2.6.A . The highest (Tumour.CFU-S) count of 45 colonies was for the 1.5×10^5 cells/mouse dose, while the lowest (Tumour.CFU-S) count of 8.2 colonies was for the 5×10^4 cells/mouse dose. The 7.5×10^4 and 1×10^5 cell doses had intermediate colony contents of 15 and 20 respectively.

In another series of experiments, several duplicate trials were made for the doses 1×10^5 and 1.5×10^5 cells injected into mice, to assess the respective (Tumour.CFU-S) content for the two doses. The results were variable, the 1×10^5 cell dose gave the following range for mean (Tumour.CFU-S) content = 14.7 - 27.3 - 54.72 with a new result of 48 colonies as shown in Table 2.6.B. For the 1.5×10^5 cell dose, the mean (Tumour.CFU-S) content ranged from 36.75 - >60 colonies per spleen, (too many to be counted), and in Table 2.6.A the mean (Tumour.CFU-S) content for 1.5×10^5 cell dose was 45.

Therefore, it was decided for experiment 2.6.B to choose a cell dose, that does not give a high colony count such as the 1.5×10^5 cell dose/mouse which renders the colony counting difficult.

The dose used for all experimental treatments described in Table 2.6.B was 1×10^5 cells/mouse . Mice which received the plasmacytoma suspension which was without UFF or Ara/C (Control animals) had the highest (Tumour.CFU-S) content of 48 colonies per

Table 2.6.A: The number of tumour colony forming units-spleen(T.CFU-S)per spleen, per dose of plasmacytoma cells. injected intravenously into mice, the number of colonies(T.CFU-S)has been expressed as mean (T.CFU-S)with ± S.E. included.

Cell dose per mouse	Mean number of (CFU-S) per spleen ± S.E.	number of animals surviving at day 9
5×10^4	8.2 ± 1	A 14/20, B 3/20
7.5×10^4	15.1 ± 3.4	A 20/20, B 8/20
1×10^5	20.3 ± 2	A 20/20, B 10/20
1.5×10^5	45.0 ± 6	A 15/20, B 11/20

A = 1st experiment.
B = 2nd experiment.

Table 2.6.B: The effect of UIF or Ara/c treatments on plasmacytoma cultures, cultured in vitro, on their colony forming ability (CFU-S) in the spleens of mice. The values are expressed as mean colony number per spleen with ± standard error included.

Experimental treatment	Mean (CFU-S) per spleen per 1 x 10^5 cells injected ± S.E.	Number of animals surviving at day 9
UIF	9.4 ± 0	A 11/20, B 7/20
UIF - Ara/c	11.14± 0	A 11/20, B 5/20
Ara/c	38.0 ± 1	A 9/20, B 6/20
Controls without UIF or Ara/c	48.0 ± 2	A 7/20, B 8/20

A = 1st experiment.
B = 2nd experiment.

spleen. Mice which received the plasmacytoma suspension which was treated with Ara/C, had a mean colony content of 38. Thus the percentage of cells that were in cycle and susceptible to the effects of Ara/C, equals 20.83%.

For plasmacytoma cultures, treated with UIF and Ara/C the mean number of colonies per spleen droped to 11, while cells treated with UIF without Ara/C gave a mean colony content of 9.4 which represented an 80.41% reduction in mean colony content as compared to normal controls values.

The difference in mean colony content between the Ara/C and UIF-Ara/C groups was significant, ($P<.01$, $t=5.87$), while the difference between the UIF and UIF-Ara/C was not significant, ($t=0.43$, $P>.05$). The difference between the UIF and control (without UIF) groups was significant, ($t=18.16$, $P<.01$), and the difference between the Ara/C and control (without UIF) groups was also significant, ($t=2.39$, $P=0.030$ or $0.05>P>0.02$).

As described earlier, (section 3.2.3) the inhibitory effects of UIF on cell cultures were reversible, and STD UIF was not cytotoxic or having a lasting inhibitory effect on DNA synthesis.

Similarly, (section 3.2.4) the inhibitory effects of UIF were not evident on the passage of cells through S phase, and the metaphase count was similar to that of control cultures.

Similarly, (section 3.2.5) the labelling index of plasmacytoma cultures was similar to control cultures. The grain count distribution may suggest that UIF was competing with radioisotope for DNA as was evident in the densely labelled group. However, this was not evident in the other two groups. As judged by the above mentioned results (together with the cell viability tests), UIF therefore, had no significant effect on the proliferation of plasmacytoma cultures, (see below).

The results described above, (section 3.2.6) have demonstrated that the percentage of cells in S phase was approximately 21%. The results have also demonstrated that with this method of cell proliferation assessment (colony formation-spleen) UIF proved to be inhibitory for cell proliferation as the colony content of spleens from UIF treated cultures, was the lowest among other counterpart cultures especially that of Ara/C, which is an S phase specific cytotoxic drug.

3.2.7: The effects of UIF ultrafiltrate fractions on the uptake of 125 IUDR by plasmacytoma cells.

Experimental procedures:

Rat STD.UIF was prepared as described previously and was divided into 3 groups. The first group was normal STD.UIF, the second group was heated for ½ hour at 56°C in a water bath, and the third group was fractioned in an Amicon (Amicon - UK) Diaflo ultrafiltration apparatus. The Diaflo ultrafiltration procedures, fractioned the UIF into 5 molecular weight groups: Above 5×10^{4} = fraction 5×10^{4}, Above 1×10^{4} and below 5×10^{4} = fraction 10^{4}, Above 1×10^{3} and below 1×10^{4} = fraction 10^{3}, Above 5×10^{2} and below 1×10^{3} = fraction A500, and below 5×10^{2} = fraction B500, as described in materials and methods.

A plasmacytoma suspension was prepared with and without 10% FCS, and was dispensed at 1×10^{5} cells per microtitre well of each suspension. 40 ul of each type of UIF were dispensed into 5 microtitre wells, together with 0.2 uci 125 IUDR per well. The final volume per well was 200 ul. The pulsing procedure was as follows: 0/4, 2/4 and 4/4. In the 0/4 incubation sequence, the UIF and the 125 IUDR were dispensed at zero time, together, to the cell suspension and incubated for 4 hours at 37°C. In the 2/4 incubation sequence, the UIF was added to the cultures and incubated for 2 hours at 37°C, followed by the addition of 125 IUDR and a further incubation of 4 hours at 37°C. In the 4/4 incubation sequence, the UIF was added to the cultures and incubated for 4 hours at 37°C, followed by the addition of 125 IUDR and a further incubation of 4 hours at 37°C.

Results:

In the 0/4 incubation group, the highest inhibition of isotope uptake by cells cultured without FCS were by whole UIF.STD, UIF.56°C

and amicon fraction B500 as described in Table/Figure 2.7 which were around the same region of 27 to 30% inhibition and were significantly different from their culture controls ($P<.01$), while amicon fractions A500, 10^3, 10^4, and 5×10^4 were weakly inhibitory, if any, and were not significantly different ($P>0.5$) from control cultures without UIF. For cells cultured with 10% FCS, the inhibitions of isotope uptake produced by the respective amicon fractions were similar at around the 20% region except for A500 which was around 15%. STD.UIF produced 21% inhibition while STD 56°C UIF produced a relatively higher inhibition of 30%. The respective mean Cpms of cell cultures, cultured in all UIF groups were significantly different from control cultures mean Cpm ($P<.01$ and $0.05>P>0.1$) as described in the t-test comparison for Table 2.7. All UIF fractions produced more inhibitions in 10% FCS and were significantly different from their counterpart cultures without FCS ($P<.01$) except for B500 which was not significantly different. Similarly the inhibitions produced by STD.UIF 56°C were similar in both cultures, $P>.05$, while for STD.UIF there was a drop in inhibition of about 6% in 10% FCS cultures which was significant $0.05>P>.01$.

In the 2/4 group (without FCS) the highest inhibition of isotope uptake by cell cultures, was produced by amicon fraction B500 at 28% followed by a nearly similar inhibition by both UIFS.STD and 56°C at around the 20% region, which were all signficantly different from control cultures ($P<.01$) as described in the t-test comparison of table 2.7. Similarly, as in the 0/4 group the least inhibitory were amicon fractions A500, 10^3, 10^4, and 5×10^4 which were within the 4-6% inhibition. However, due to their small standard errors in terms of the relatively large Cpm value for each fraction including control cultures, they were significantly different from control cultures

(P<.01 and 0.05>P>0.1) as described in the t-test comparison of Table 2.7.

The inhibitions of radioisotope uptake produced in 10% FCS cultures by the respective UIFS or amicon fractions, was highest for STD.UIF at 27%, followed by a similar inhibition of 23% by UIF.56 °C, while B500 was relatively smaller at 16%; all of which were significantly different from the uptake of radioisotope by control cultures (P <.01). The least inhibitory, were UIF amicon fractions: A500, 10^3, 10^4 and 5 x 10^4, with an inhibition range of 4 to 5%, all of which, were not significantly different from the uptake of radioisotope by control cultures (P >.05), except for fraction 10^3 which was different (0.05>P>0.01) due to its relatively low cpm and standard error in terms of the value of control cultures. The respective mean percent inhibitions in 10% FCS cultures were not significantly different from their counterpart cultures, cultured without FCS (P>.05), except for B500 which was more inhibitory in cultures without FCS , than in cultures containing 10% FCS.

In the 4/4 incubation sequence culture group (without FCS), the highest inhibition of radioisotope uptake by cell cultures was produced by amicon fraction B500, at around 34%, followed by STD.UIF, at around half as less to 17%, while its variant, UIF.56 °C, at a slightly higher inhibition of 22%; all of which were significantly different from the uptake of radioisotope by control cultures (P<.01). Again amicon fractions A500, 10^3, 10^4 and 5 x 10^4 were least inhibitory, with a range of 3 to 9% inhibition, of which, the mean cpm of fractions A500 and 10^4 were not significantly different from the mean cpm of control cultures, where as the mean cpm of fractions 10^3 and 5 x 10^4 were different (0.05>P>.01 and

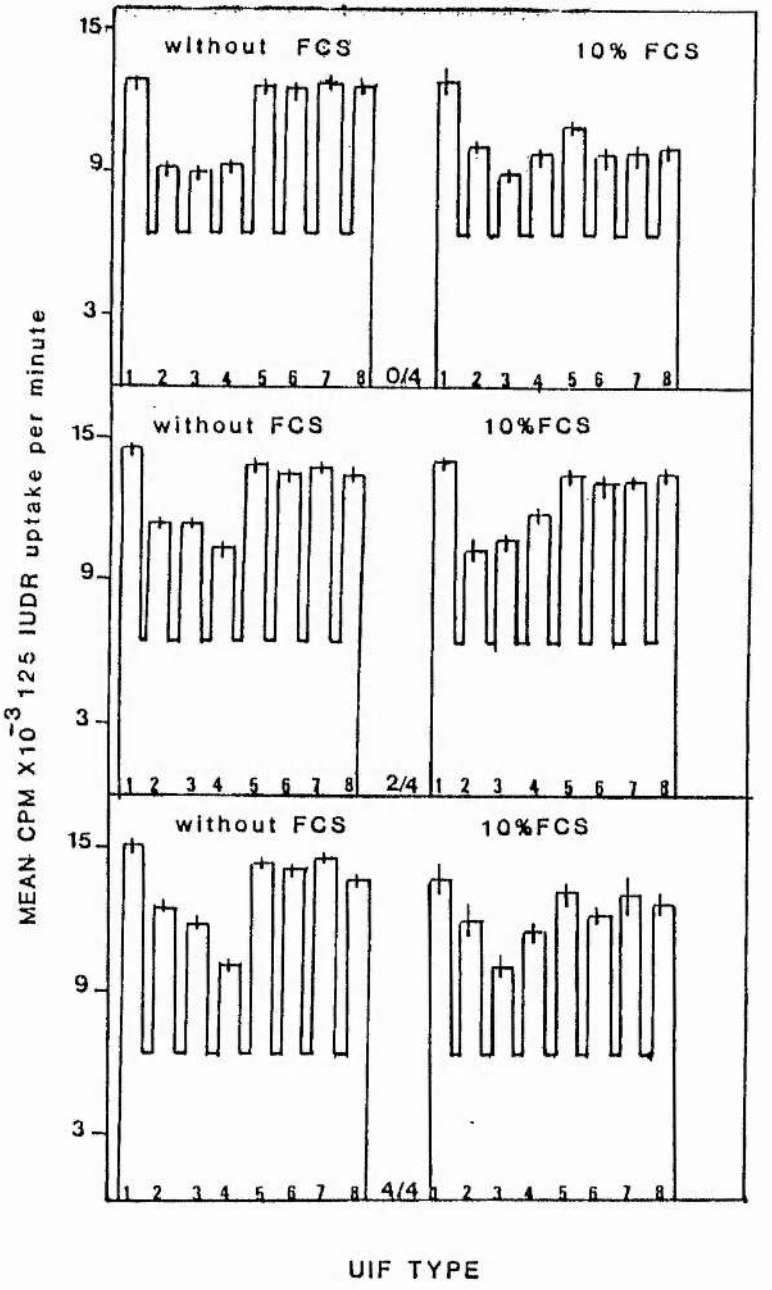


FIGURE 2.7: The uptake of 125IUDR by plasmacytoma cultures, cultured without and with FCS-HI, for 4 hours with different fractions of rat standard (STD) UIF. The incubation sequence for the respective incubations was: 0/4, 2/4 and 4/4 . **UIF concentration** = 20 %
1: control cultures without UIF. 2: STD UIF. 3: STD UIF heated 56c, 30 min.
4: B500. 5: A500. 6: 10^3. 7: 10^4. 8: 5×10^4. ***
***:see table 2.7 for details.

Table 2.7: The effect of amicon ultra filtrate fractions of UIF on the uptake of 125IUDR by plasmacytoma cultures. The values are expressed as mean counts per minute (cpm) and as percent inhibition. The ± standard error is included.

The cultures were incubated for 0/4, 2/4 and 4/4 hours incubation periods.

0/4 hours incubation sequence.

	Without 10% FCS		With 10% FCS	
UIF Fraction	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
5×10^4	12542 ± 331	2.07 ± 2.5	10046 ± 589	21.78 ± 4.5
10^4	12772 ± 235	0.2 ± 1.8	9917 ± 344	22.79 ± 2.6
10^3	12388 ± 227	3.2 ± 1.7	9773 ± 130	23.90 ± 1
A 500	12655 ± 188	1.1. ± 1.4	10945 ± 307	14.78 ± 2.3
B 500	9204 ± 206	28.13 ± 1.6	9808 ± 213	23.63 ± 1.6
56°C	8907 ± 177	30.45 ± 1.3	8964 ± 198	30.20 ± 1.5
STD	9249 ± 97	27.78 ± 0	10024 ± 253	21.90 ± 1.9
Controls	12807 ± 148	0.00	12844 ± 656	0.00
2/4 hours incubation sequence				
5×10^4	13514 ± 121	6.77 ± 0	13418 ± 149	4.2 ± 1
10^4	13865 ± 127	4.30 ± 0	13361 ± 385	4.6 ± 2.7
10^3	13575 ± 199	6.30 ± 1.3	13184 ± 176	5.9 ± 1.2
A 500	13964 ± 135	3.60 ± 1.4	13435 ± 234	4.1 ± 4.6
B 500	10402 ± 163	28.20 ± 1.1	11743 ± 258	16.2 ± 1.8
56°C	11456 ± 310	20.97 ± 2.1	10766 ± 254	23.19± 1.8
STD	11445 ± 263	21.05 ± 1.8	10178 ± 437	27.39± 3.1
Controls	14496 ± 100	0.00	14018 ± 305	0.00

Table 2.7. (Continued)

4/4 hours incubation sequence

	Without 10% FCS		With 10% FCS	
UIF Fraction	mean cpm ± S.E.	Percent Inhibition ± S.E.	mean cpm ± S.E.	Percent Inhibition ± S.E.
5×10^4	13759 ± 128	8.8 ± 0	12554 ± 592	8.3 ± 4.3
10^4	14638 ± 160	3.04 ± 1	12984 ± 848	5.2 ± 6.1
10^3	14184 ± 204	6.04 ± 1.3	12147 ± 259	11.3 ± 1.8
A 500	14402 ± 219	4.6 ± 1.4	13214 ± 630	3.5 ± 4.6
B 500	10013 ± 229	33.6 ± 1.5	11644 ± 404	14.9 ± 2.9
56°C	11852 ± 414	21.5 ± 2.7	10138 ± 724	25.9 ± 5.2
STD	12465 ± 212	17.42 ± 1.4	11975 ± 761	12.5 ± 5.5
Controls	15097 ± 222	0.00	13697 ± 627	0.00

STD = Standard UIF; 56C = STD,UIF heated for 30 min at 56°C.

Amicon molecular weight fractions: $5 \times 10^4 = > 5 \times 10^4$, $10^4 = 5 \times 10^4 >$ molec.wt. $> 10^4$, $10^3 = 10^4 >$ molec.wt. $> 10^3$, A 500 = $10^3 >$ molec.wt. $> 5 \times 10^2$, B 500 < 500.

T-test Table for Table 2.7.

(1) = Without FCS, (2) = With 10% FCS

0/4 Group

UIF.VS. Controls (1)		UIF.VS. Controls (2)	UIF.VS.STD.UIF. (1)	UIF.VS.STD.UIF. (2)
5×10^4 =	p*	p*/*	p**	p*
10^4 =	p*	p**	p**	p*
10^3 =	p*	p**	p**	p*
A 500 =	p*	p*/*	p**	p*/*
B 500 =	p**	p**	p*	p*
56C =	p**	p**	p*	p**
STD =	p**	p**		
2/4 Group				
5×10^4 =	p**	p*	p**	p**
10^4 =	p**	p*	p**	p**
10^3 =	p**	p*/*	p**	p**
A 500 =	p*/*	p*	p**	p**
B 500 =	p**	p**	p**	p*/*
56C =	p**	p**	p*	p*
STD =	p**	p**		

T-test Table for Table 2.7. (continued)

(1) = Without FCS, (2) = With 10% FCS

4/4 Group

UIF.VS. Controls (1)		UIF.VS. Controls (2)	UIF.VS.STD.UIF (1)	UIF.VS.STD.UIF. (2)
5×10^4 =	p**	p*	p**	p*
10^4 =	p*	p*	p**	p*
10^3 =	p*/*	p*	p**	p*
A 500 =	p*	p*	p**	p*
B 500 =	p**	p* *	p**	p*
56C =	p**	p**	p*	p*
STD =	p**	p*		

T-test Table for Table 2.7 (UIF-FCS).VS.(UIF - Without FCS)

0/4 Group		2/4 Group	4/4 Group
5×10^4 =	p**	p*	p*
10^4 =	p**	p*	p*
10^3 =	p**	p*	p*
A 500 =	p**	p*	p*
B 500 =	p**	p**	p**
56C =	p*	p*	p*
STD =	p*/*	p*	p*

$p^{*/*} = .05 > P > .01$

$p^{**} = < .01$

$p^{*} = > .05$

P<.01) respectively. For cells cultured with 10% FCS, the highest inhibition of radioisotope uptake was produced by UIF.56 ^{0}C, at approximately 26%, followed by STD.UIF at half as less inhibition of around 13%, with a slight increase of 2% inhibition by B500, to 15%.

Amicon fraction 10^3 produced 11% inhibition, the 5 x 10^4 fraction produced 8% inhibition, while the A500 and 10^4 fractions were between 3 to 5% inhibitory. The mean cpm of cell cultures , cultured in all the respective UIFS (and fractions), were not significantly different from the mean cpm of control cultures (P>.05), except for B500 and UIF.56 ^{0}C, which were different (0.05>P>.01 and P<.01) respectively.The differences in the mean percent inhibitions of radioisotope uptake between cultures cultured with the respective UIFS, in the presence or absence of FCS, were not significant for all UIFS (P>.05), except for B500, which produced higher inhibitions when cultured without FCS, than its counterpart culture which was co-cultured with FCS (P<.01).

Comparing the mean percent inhibitions of radioisotope uptake of the respective UIFS (and fractions) in terms of the inhibition of radioisotope uptake produced by STD.UIF, the following results were obtained:

In the 0/4 incubation culture group (without FCS), UIF fractions 5 x 10^4, 10^4, 10^3 and 500 were all significantly different from STD.UIF, while UIF fraction B500 and UIF.56 ^{0}C were not. In cultures cultured with 10% FCS, all UIF fractions were not significantly different from STD.UIF, except for fraction A500, and UIF.56 ^{0}C, which were different as described in the t-test comparison of Table 2.7.

In the 2/4 culture group (without FCS) and with (10% FCS), all fractions were significantly different from STD.UIF, except UIF.56 ^{0}C, which was not different.

In the 4/4 culture group (without FCS), all fractions were significantly different from STD.UIF, except UIF.56 ^{0}C, which was not. In cultures cultured with 10% FCS, all fractions including UIF.56 ^{0}C, were not significantly different from STD.UIF as described in t-test comparison of Table 2.7.

In the 0/4 culture incubation group, the mean cpm of control cultures (with and without FCS) were similar (P>.05).

In the 2/4 incubation group, the mean cpm of control cultures cultured with 10% FCS were slightly lower than the mean cpm of control cultures, cultured without FCS by 3.9%, which was not a significant difference (P>.05).

In the 4/4 culture group, the mean cpm of control cultures cultured with 10% FCS was less by 9.26% than the mean cpm of contol cultures, cultured without FCS, which was not a significant difference (P>.05), but may signal that the inhibition of radioisotope uptake by cell cultures may be increasing as the culture incubation sequence is changed to longer incubation sequences.

As described above,(section 3.2.7) the effects of various fractions of UIF on the uptake of radioisotope by cell cultures with respect to the incubation squence and the presence of FCS in cultures have been assessed.

The results have demonstrated that FCS and the incubation sequence may influence the uptake. Thus for fractions 5 x 10^4, 10^4, 10^3 and A500, the inhibition of radioisotope uptake (0/4 without FCS) was barely evident, while for the same fractions (cultured with FCS) the inhibition of radioisotope uptake was quite effective. However, at 2/4 and 4/4 incubation sequences the effects of the above mentioned fractions (with and without FCS) were minimal and the respective radioisotope uptake was not significantly different from that of control cultures.

The most inhibitory fraction among the UIF fractions was the B500 fraction, which suggests that the active inhibitory substance was of small molecular weight. Similarly, the effects of FCS on B500 were not potentiating, as the inhibition of radioisotope uptake (with FCS) in the 2/4 and 4/4 sequences were reduced relative to the inhibition in serum free cultures. The heat inactivated UIF was also inhibitory (more inhibitory than non heat treated STD UIF) which suggested that some constituents of UIF which produced the inhibition were heat resistant. It was also noticed that the inhibitory effect of UIF 56°C remained stable in both FCS environments.

3.3. IN VITRO CULTURES: ASSESSMENT OF THE EFFECTS OF POLYAMINES ON CELL PROLIFERATION.

3.3.1.1: The effects of spermine and spermidine which were preincubated with FCS or human serum for 16 hours at room temperature on the uptake of 125IUDR by cell cultures.

Experimental procedures:

Several concentrations of spermine and spermidine were prepared and mixed with FCS in the proportion of 100ul spermine or spermidine to 20 ul FCS (100ul polyamine: 20 ul FCS) for 16 hours at room temperature. The following cell suspensions were prepared in RPMI 1640: plasmacytoma (PC), mammary adenocarcinoma (M.Aden), normal rat thymocytes (RTC), and normal mouse thymocytes (MTC). Each cell suspension was dispensed into tissue culture microtitre wells at 1×10^5 cells/well. The final concentrations of spermine and spermidine per 200 ul final volume per well were as follows: 8ug, 4ug, 2ug, 0.8ug, 0.4ug, 0.08ug and 0.04ug.

The following additions of the polyamine incubates were added to microtitre wells

120ul incubate [100ul polyamine+20ul FCS]+60ul cell suspension + 20ul125IUDR

120ul incubate[100ul polyamine + 20ul RPMI)+60ul cell suspension+20ul125IUDR

Control cultures for the above cultures contained no polyamines. Each cell suspension was cultured independently in separate microtitre culture plates with 0.2 uci of 125IUDR and pulsed for 4 hours at 37 °C, 5% CO_2. Five replicate cultures for each polyamine

concentration were prepared. The uptake of isotope by cultures was assessed as described previously.

In a similar experiment, human serum was used instead of FCS and the procedure of the experiment was similar to the one described above for the FCS group, but with the following concentrations of polyamine per well,: 8ug, 4ug, and 0.8ug. The cell cultures were also similar, apart from the mouse thymocytes which were not included. The culture incubation sequence was 0/4 hours , i.e. the polyamine incubate was added at the same time as 125IUDR to the cell cultures. The incubation sequence of the above mentioned cultures was as follows: 16 PC. 0/4, 16 M.Aden. 0/4, 16 RTC. 0/4, 16 MTC. 0/4 .

Results:

The spermidine-FCS and the spermine-FCS reactions produced a linear dose response effect on the inhibition of uptake of 125IUDR by plasmacytoma cultures, (Figure/Table 3.1.1A. PC.0/4 incubation sequence) at 0.8 to 8ug/well concentrations. The inhibitions were similar, and were in the regions of 40 and 90% respectively. The inhibition of isotope uptake in both cultures was linear and dose dependent. The mean cpm of the respective cultures was significantly different from the mean cpm of control cultures, ($P<.01$). The ID50 (polyamine-FCS) for spermidine and spermine was approximately similar, and was 16.96 and 20.10uM respectively, (Table 3).

For cultures, cultured without FCS, the uptake of isotope at the respective polyamine concentrations was neither linear nor dose dependent. No significant inhibition of isotope was observed, ($P>.05$) and the general trend of isotope uptake was higher in

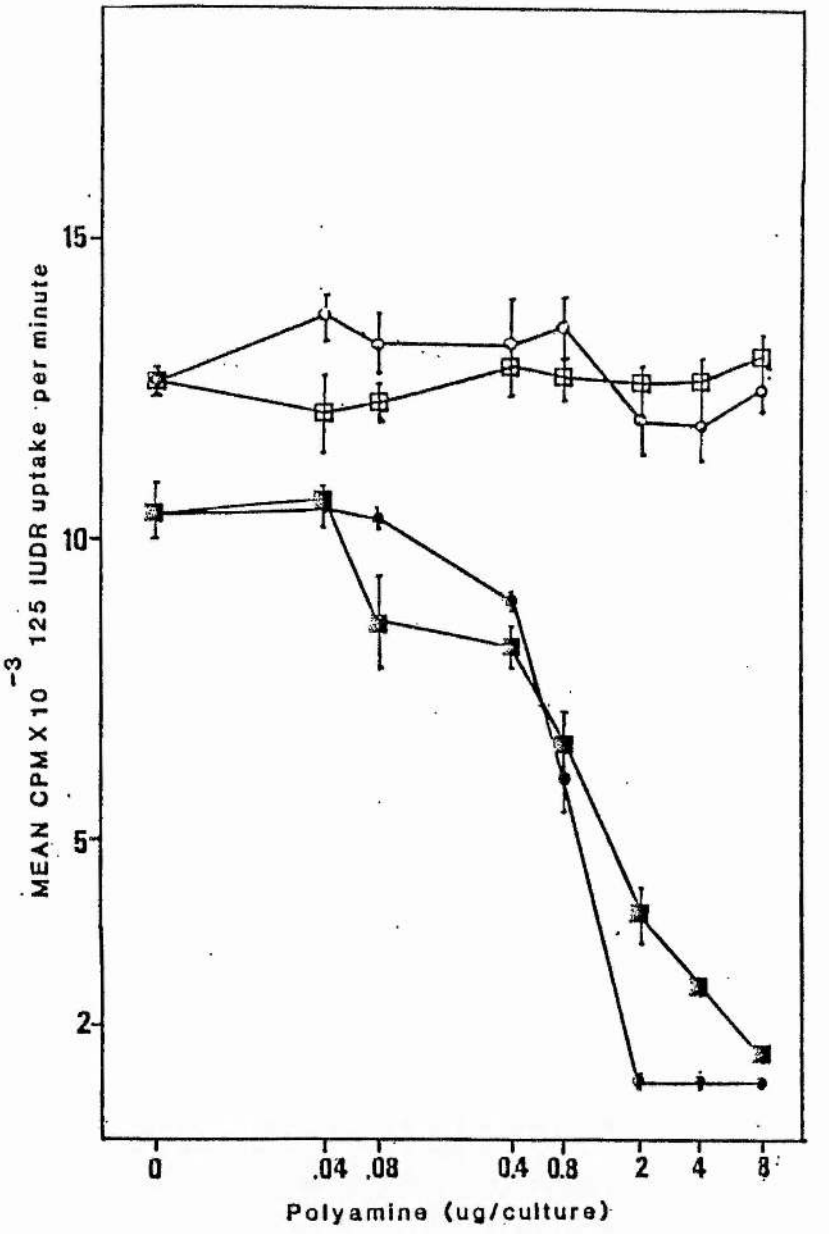


FIGURE 3.1.1A: The uptake of 125IUDR by plasmacytoma cultures, cultured for 4 hours with different concentrations of polyamines per culture. 0/4 incubation sequence.
without FCS: spermidine o—o , spermine □—□
with FCS-HI: spermidine ●—● , spermine ■—■

TABLE 3.1.1A: The effects of spermidine and spermine on the uptake of ^{125}IUDR by plasmacytoma cultures, cultured with or without 10% FCS for 0/4 hours. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± standard error is included.
SD = spermidine, SM = spermine.

polyamine conc. µ/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	918 ± 44	1473 ± 75	91.24 ± 0.45	85.95 ± 0.71	12550 ± 430	13089 ± 367	1.24 ± 3.27	+2.99 ± 2.9
4	928 ± 84	2557 ± 97	91.14 ± 0.1	75.62 ± 0.92	11912 ± 393	12650 ± 304	6.2 ± 3.19	0.45 ± 2.4
2	935 ± 61	3762 ± 390	91.08 ± 1.31	64.13 ± 3.72	12361 ± 608	12659 ± 150	2.72 ± 4.79	0.38 ± 1.53
0.8	5983 ± 294	6560 ± 517	42.95 ± 2.8	37.45 ± 4.93	13524 ± 351	12752 ± 320	+6.42 ± 2.67	0.34 ± 2.82
0.4	8976 ± 114	8266 ± 288	14.42 ± 1.08	21.19 ± 3.54	13264 ± 775	12970 ± 512	+4.37 ± 6.1	+2.05 ± 4.04
0.08	10398 ± 157	9514 ± 743	0.86 ± 1.5	9.28 ± 7.09	13269 ± 543	12322 ± 278	+4.41 ± 4.28	3.04 ± 2.19
0.04	10547 ± 248	10620 ± 171	+0.55 ± 2.37	1.24 ± 1.6	13699 ± 284	12133 ± 642	+7.79 ± 2.24	4.52 ± 5.06
control	10489 ± 515				12708 ± 162			

+ = stimulation of isotope uptake

cultures cultured without FCS. The mean cpm of the respective cultures was not significantly different from controls, (P>.05). The inhibitory effects of polyamines in the presence of FCS contrasts with the effects of polyamines when incubated without FCS.

The uptake of 125IUDR by mammary adenocarcinoma was inhibited by spermidine-FCS and spermine-FCS with a linear dose-response effect, (Figure/Table 3.1.1B)(M.Aden. 0/4 incubation sequence). For both spermidine and spermine the inhibition of isotope uptake at the 0.8 to 8ug/well concentrations was similar, and was in the region of 50-60 to 80% respectively. The mean cpm of the respective cultures was significantly different from the mean cpm of control cultures, (P<.01). At higher dilutions of the respective polyamines, (0.8ug) the mammary adenocarcinoma was more sensitive to the inhibitory effects of the polyamine FCS reaction, than the plasmacytoma.

The ID50 (polyamine-FCS) for mammary adenocarcinoma cultured with spermidine and spermine, was 16.81 and 7.58uM respectively, (Table 3).

The uptake of isotope by mammary adenocarcinoma cultured with the respective polyamines (without FCS), was neither linear nor dose dependent, and was not significantly different from the uptake of isotope by control cultures, (P>.05). The pattern of uptake in these cultures, (polyamine without FCS) is in contrast to the pattern observed in cultures cultured with polyamines and FCS, in which there was a marked inhibition of isotope uptake.

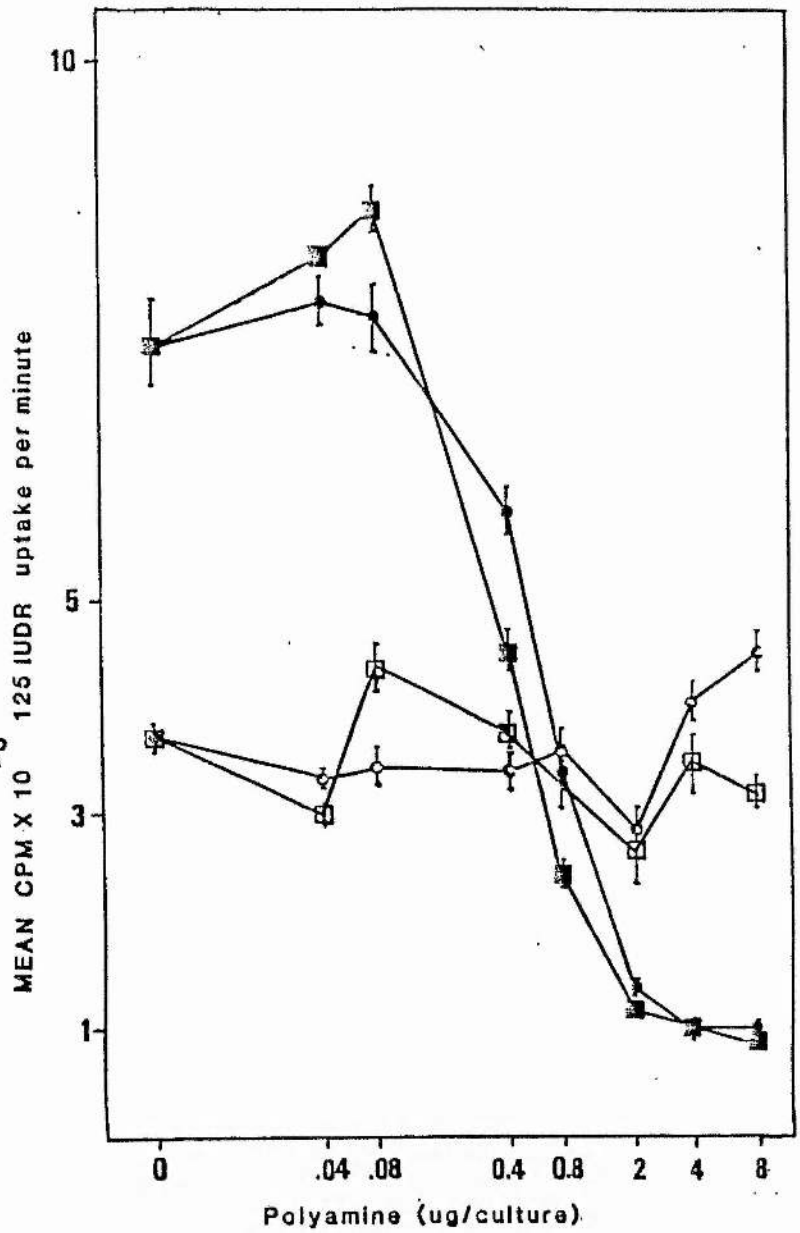


FIGURE 3.1.1B: The uptake of 125IUDR by mammary adenocarcinoma cultures, cultured for 4 hours with different concentrations of polyamines per culture. 0/4 incubation sequence.
without FCS: spermidine o—o , spermine □—□
with FCS-HI: spermidine •—• , spermine ■—■

TABLE 3.1.1B : The effects of spermidine and spermine on the uptake of ^{125}IUDR by mammary adenomacarcinoma cultures, cultured with or without 10% FCS for 0/4 hours. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± standard error is included.
SD = spermidine, SM = spermine.

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	100 ± 5	89 ± 3	86.32 ± 0.68	87.99 ± 0.37	455 ± 7	320 ± 14	+22.70 ± 2.24	13.4 ± 3.8
4	102 ± 8	101 ± 6	86.04 ± 1.16	86.1 ± 0.80	284 ± 17	352 ± 20	23.17 ± 4.56	4.88 ± 5.65
2	142 ± 9	118 ± 5	80.57 ± 1.3	83.83 ± 0.6	354 ± 13	266 ± 22	4.36 ± 3.46	28.27 ± 6.25
0.8	339 ± 22	243 ± 12	53.57 ± 3.18	66.63 ± 1.6	338 ± 22	318 ± 17	8.63 ± 6.01	14.23 ± 4.60
0.4	578 ± 19	451 ± 20	20.82 ± 2.72	38.23 ± 2.86	342 ± 13	378 ± 23	7.69 ± 3.79	+1.96 ± 6.30
0.08	762 ± 32	861 ± 14	+4.36 ± 4.49	+17.86 ± 1.89	330 ± 19	436 ± 30	10.76 ± 5.28	+17.6 ± 8.38
0.04	778 ± 24	818 ± 13	+6.42 ± 1.89	+11.99 ± 1.75	380 ± 6	301 ± 15	+2.59 ○ 1.69	18.57 ± 3.96
control	731 ± 42				370 ± 9			

+ = stimulation of uptake

The polyamine-FCS reaction produced a linear dose-response inhibition on the uptake of 125IUDR by rat and mouse thymocytes, [(Figures/Tables 3.1.1C and 1D)(RTC. and MTC. 0/4 incubation sequence)].

The inhibition of isotope uptake by spermidine and spermine (0.8 to 8ug per well concentrations), on rat and mouse thymocytes cultured with FCS, was similar. The inhibitions were in the regions of 80 and 90% respctively, (Figures/Tables 3.1.1C and 1D). The mean cpm of the respective cultures was significantly different from the mean cpm of control cultures, ($P<.01$). The rat and mouse thymocytes were more sensitive (approximately twice as sensitive) to the inhibitory effects of polyamine-FCS than either the plasmacytoma or mammary adenocarcinoma, at higher dilutions (0.4ug) of the respective polyamines.

The ID50 for rat and mouse thymocytes (spermidine-FCS), was similar, and was 5.02 and 6.28 uM respectively. The ID50 (spermine-FCS) was also similar for both sets of cultures, and was 3.67 and 2.87 uM respectively, (Table 3). The ID50 of both sets of cultures was less than that of plasmacytoma and mammary adenocarcinoma.

For both sets of cultures, cultured with the respective polyamines (without FCS), the uptake of isotope was neither linear nor dose dependent, and this contrasts with the inhibitory effects produced by the respective polyamines on cultures cultured with FCS.

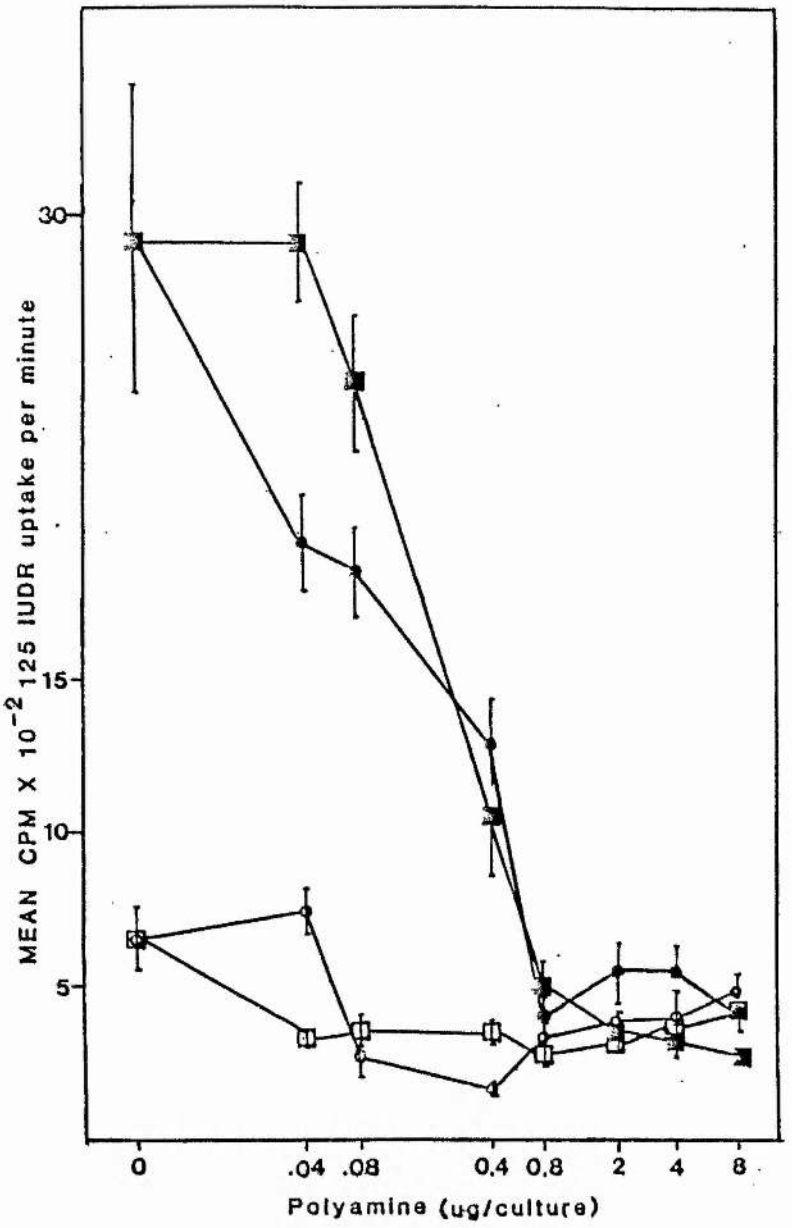


FIGURE 3.1.1C: The uptake of 125IUDR by rat thymocyte cultures, cultured for 4 hours with different concentrations of polyamines per culture. 0/4 incubation sequence .
without FCS: spermidine o—o , spermine □—□
with FCS-HI: spermidine •—• , spermine ■—■

TABLE 3.1.1C: The effects of spermidine and spermine on the uptake of ^{125}IUDR by rat thymocyte cultures, cultured with or without 10% FCS for 0/4 hours. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± standard error is included.
SD = spermidine, SM = spermine.

polyamine conc. µg/well	with 10% FCS				without 10% FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	41 ± 11	27 ± 2	85.86 ± 3.96	90.68 ± 0.65	47 ± 5	44 ± 9	26.56 ± 14.26	31.25 ± 7.93
4	54 ± 7	30 ± 5	81.37 ± 2.75	89.25 ± 1.86	39 ± 7	38 ± 6	39.06 ± 10.52	40.62 ± 12.26
2	55 ± 10	34 ± 3	81.03 ± 3.56	88.27 ± 1.07	31 ± 2	34 ± 4	52.21 ± 7.62	46.87 ± 4.07
0.8	39 ± 7	49 ± 8	86.39 ± 2.51	82.88 ± 2.95	32 ± 5	29 ± 1	50.00 ± 2.68	55.32 ± 7.60
0.4	127 ± 14	104 ± 19	56.20 ± 5.16	64.07 ± 6.63	16 ± 1	34 ± 4	75.0 ± 6.98	46.87 ± 2.21
0.08	186 ± 14	246 ± 21	35.86 ± 4.79	15.1 ± 7.22	27 ± 7	34 ± 4	58.2 ± 6.76	46.87 ± 11.14
0.04	193 ± 18	291 ± 20	33.44 ± 6.29	+0.34 ± 6.93	30 ± 7	33 ± 3	53.34 ± 5.41	48.45 ± 11.10
control	290 ± 71				64 ± 9			

+ = stimulation of uptake

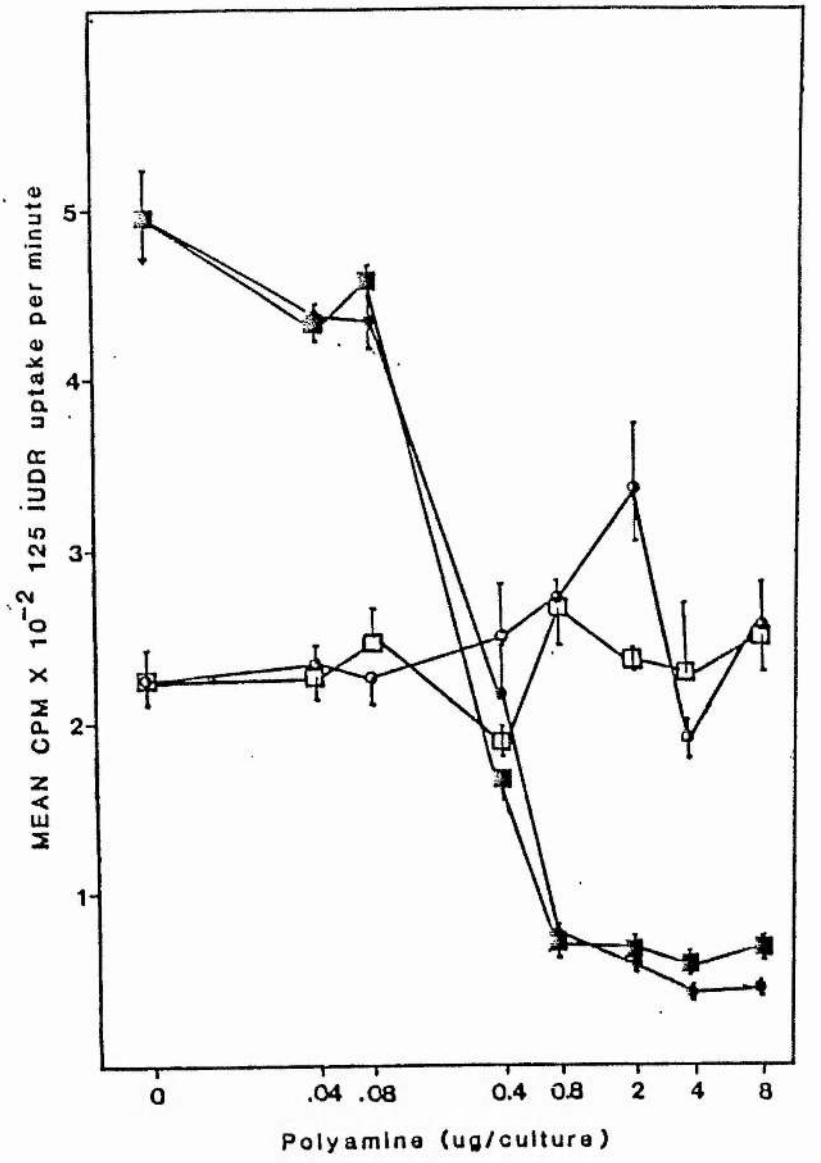


FIGURE 3.1.1D: The uptake of 125IUDR by mouse thymocyte cultures, cultured with different concentrations of polyamines per culture, for a 4 hours incubation period. 0/4 incubation sequence.
without FCS: spermidine o—o, spermine □—□
with FCS-HI: spermidine •—•, spermine ■—■

TABLE 3.1.10: The effects of spermidine and spermine on the uptake of ^{125}IUDR by mouse thymocyte cultures, cultured with or without 10% FCS for 0/4 hours. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The standard error is included.
SD = spermidine, SM = spermine.

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	46 ± 6	69 ± 8	90.66 ± 1.38	86 86 ± 2.49	257 ± 24	251 ± 21	+13.71 ± 10.96	+11.06 ± 7.54
4	44 ± 7	62 ± 9	91.07 ± 1.42	87.44 ± 1.94	192 ± 11	230 ± 48	+15.04 ± 5.19	+ 1.76 ± 1.79
2	59 ± 8	68 ± 6	88.03 ± 1.82	86.2 ± 1.21	336 ± 32	236 ± 5	+48.67 ± 14.56	+ 4.42 ± 2.22
0.8	76 ± 6	74 ± 6	84.48 ± 1.32	85.01 ± 3.06	270 ± 8	268 ± 21	+19.46 ± 3.86	+18.58 ± 9.64
0.4	216 ± 34	167 ± 12	56.18 ± 6.93	66.13 ± 4.48	253 ± 28	193 ± 19	+11.94 ± 12.43	+14.60 ± 8.63
0.08	433 ± 19	459 ± 10	12.23 ± 4.04	6.92 ± 2.17	227 ± 23	247 ± 15	+ 0.44 ± 10.52	+ 9.29 ± 6.99
0.04	437 ± 7	433 ± 15	11.46 ± 1.46	12.17 ± 3.13	235 ± 12	228 ± 8	+ 3.98 ± 5.5	+ 0.88 ± 3.68
control	493 ± 27				226 ± 20			

+ = stimulation of uptake

The plasmacytoma, mammary adenocarcinoma and rat thymocyte cultures, were incubated with a polyamine-human serum 16 hours - room temperature incubate, together with 125IUDR for 4 hours (0/4 incubation). For plasmacytoma cultures there was a slight inhibition of 125IUDR uptake at 4ug and 8ug of spermidine at around 3-4% and for spermine at around 1-5%, (Figure/Table 3.1.1E). Their respective mean cpm was not significantly different from the mean cpm of control cultures (P>.05). For mammary adenocarcinoma and rat thymocytes there was stimulation rather than inhibition of 125IUDR uptake; both with and without human serum. Thus human serum was not as effective as FCS in its interaction with the polyamines, in producing the inhibition of isotope uptake by cell cultures.

As described above, (section 3.3.1.1: Figures/Tables 3.1.1A, 1B, 1C ,1D and 1E) the effects of interaction between the respective polyamines with serum (FCS and human serum) on the uptake of isotope by the respective cultures were assessed. The results described above have demonstrated that while FCS interacted with the respective polyamines to produce a significant inhibitory effect on the uptake of 125IUDR by the respective cell cultures , human serum was not effective in producing such an inhibition. The results have also demonstrated that spermine (FCS) was more inhibitory than spermidine (FCS) on the uptake of isotope by cell cultures. It was also demonstrated that there was a differential sensitivity among cultures in relation to the inhibitory effects of the respective polyamine-FCS. Mammary adenocarcinoma was more sensitive to the effects of spermine-FCS than plasmacytoma. The thymocytes, (normal cell types) were more sensitive to the inhibitory effects of both polyamines-FCS, than either plasmacytoma or mammary adenocarcinoma.

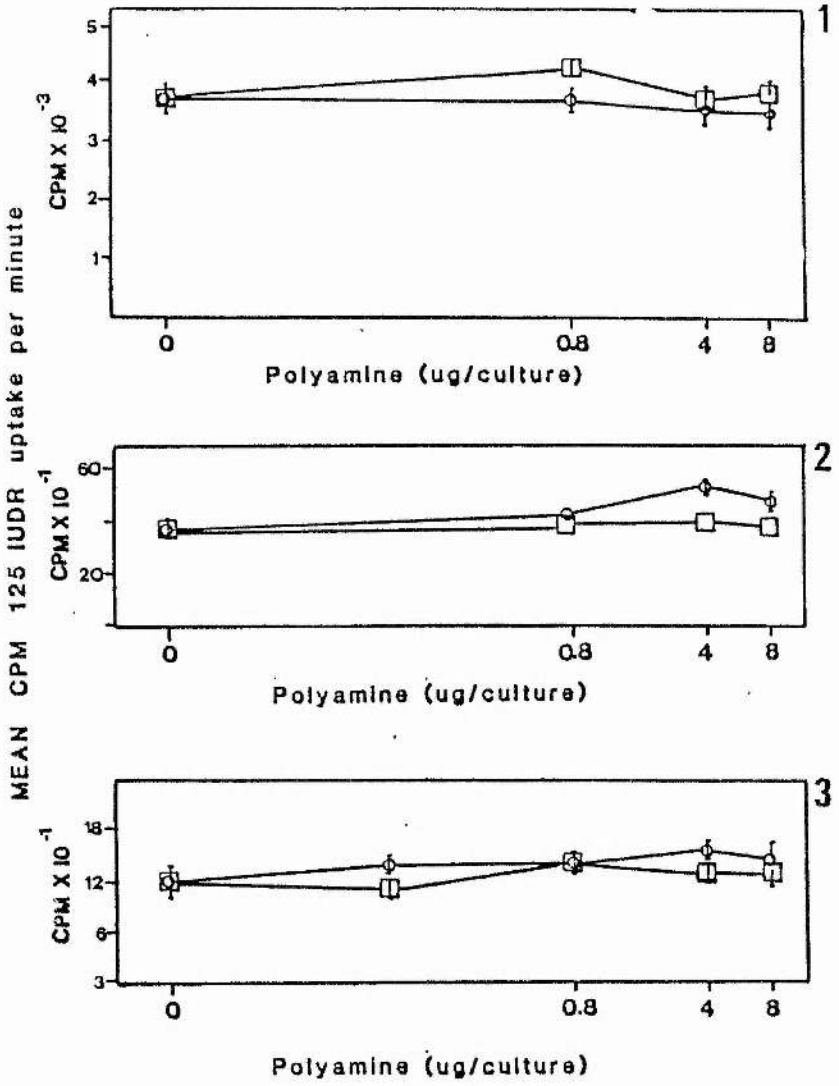


FIGURE: 3.1.1€: The uptake of 125IUDR by (1) plasmacytoma, (2) mammary adenocarcinoma and (3) rat thymocyte cultures, cultured with different concentrations of polyamines per culture for a 4 hours incubation period. 0/4 incubation sequence. Human serum spermidine o——o, spermine □——□

TABLE 3.1.1E: The effects of spermine and spermidine in the presence of 10% human serum on the uptake of 125IUDR in a 0/4 incubation sequence by the following cell cultures: plasmacytoma, mammary adenocarcinoma and rat thymocytes. The values are expressed as mean counts per minute (cpm) and as a percent inhibition. The ± standard error is included.

plasmacytoma

polyamine conc. µg/well	mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM
8	3578 ± 216	3886 ± 162	3.19 ± 5.84	5.14 ± 4.40
4	3555 ± 219	3650 ± 234	3.81 ± 5.96	1.24 ± 6.37
0.8	3704 ± 146	4282 ± 109	+0.21 ± 3.96	+15.85 ± 2.95
control	3696 ± 245			

mammary adenocarcinoma

conc. µg/well				
8	427 ± 36	368 ± 8	+16.34 ± 10.10	+0.27 ± 2.31
4	459 ± 16	378 ± 14	+25.06 ± 4.43	+4.08 ± 8.08
0.8	390 ± 11	382 ± 29	+6.26 ± 3.20	+4.08 ± 8.08
control	367 ± 22			

rat thymocytes

conc. µg/well				
8	147 ± 12	134 ± 18	+21.19 ± 10.58	+10.49 ± 15.24
4	158 ± 15	134 ± 20	+30.29 ± 13.21	+10.61 ± 16.81
0.8	141 ± 15	145 ± 11	+16.13 ± 12.72	+19.38 ± 9.05
control	121 ± 19			

SD = spermidine

SM = spermine

\+ = stimulation of uptake

3.3.1.2: The effects of polyamine incubated for 2 or 4 hours with cell cultures and FCS at 37 °C on the uptake of 125IUDR by cell cultures.

Experimental procedures: The culture procedures were similar to that described in section 3.3.1.1 with the following variation:

Polyamine and FCS were added directly to cell cultures (without preincubation of polyamine and FCS), as described earlier in section 3.3.1.1. The respective polyamine-FCS mixture (and polyamine without FCS) together with the respective cell culture were incubated for 2 or 4 hours at 37 °C, 5% CO_2 . Following the respective incubations, each culture received 20ul (0.2uci) of 125IUDR . All cultures were reincubated for a further 4 hour period at 37 °C. i.e., 2/4 and 4/4 types of incubation and pulsing sequence The cell cultures used, were plasmacytoma and normal mouse thymocytes. PC 2/4 and 4/4, TC 2/4 and 4/4.

Results:

Both spermidine and spermine in the presence of FCS produced a linear dose response inhibition on the uptake of isotope by plasmacytoma cultures, (Figures/Tables 3.1.2A and 2B. PC. 2/4 and 4/4 incubation sequences). Both polyamines (0.4ug per well) produced similar inhibitions of uptake of approximately 17%, ($P.<01$) which was significantly different from the uptake by control cultures, (Figure/Table 3.1.2A). However, the uptake inhibition curve of spermine had a relatively steeper gradient than that of spermidine, and at the 8ug/well concentration spermine produced an inhibition of 64%, while spermidine was slightly short of the 50% inhibition value. The mean cpm of both isotope uptake values was significantly ($P<.01$) different from control cultures.

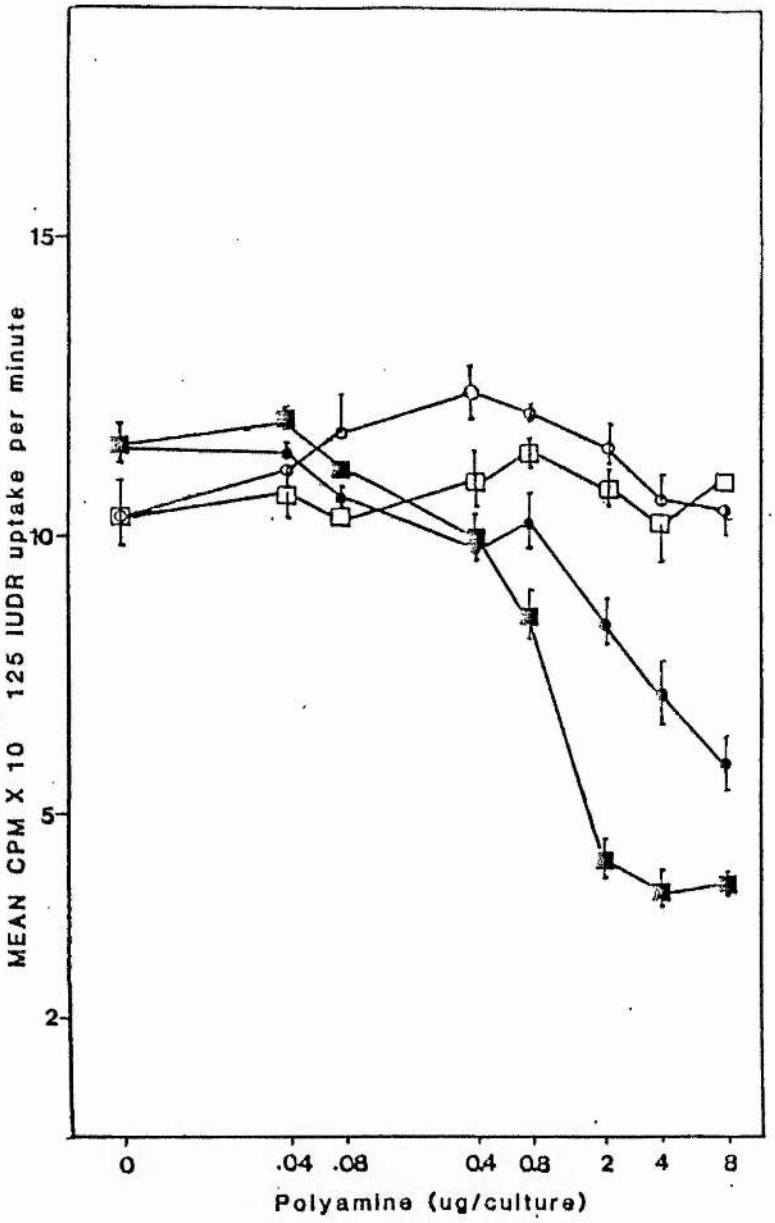


FIGURE 3.1.2A: The uptake of 125IUDR by plasmacytoma cultures, cultured for 4 hours with different concentrations of polyamines per culture. 2/4 incubation sequence .
without FCS: spermidine o——o, spermine □——□
with FCS-HI: spermidine ●——●, spermine ■——■

TABLE 3.1.2A: The effects of spermidine or spermine incubated with FCS in the presence of plasmocytoma cultures for 2 hours, on the uptake of 125 IUDR by the plasmacytoma cultures, (2/4 incubation sequence). The counts are expressed as mean counts per minute, and as percent inhibition. The ± SE is included.
SD = spermidine, SM = spermine.

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	6246 ± 477	4190 ± 144	46.99 ± 4.06	64.44 ± 1.22	10399 ± 332	10868 ± 78	0.69 ± 3.18	+3.79 ± 0.07
4	7374 ± 538	4135 ± 323	37.74 ± 3.23	64.91 ± 3.07	10161 ± 501	10659 ± 346	2.96 ± 4.80	+1.79 ± 3.31
2	8507 ± 392	4683 ± 285	27.8 ± 3.34	60.25 ± 2.42	11450 ± 3.73	10820 ± 288	+ 9.34 ± 3.57	+3.32 ± 2.76
0.8	10222 ± 437	8679 ± 408	13.25 ± 3.72	26.34 ± 3.47	12110 ± 92	11433 ± 225	+15.65 ± 0.88	+9.1 ± 2.16
0.4	9757 ± 270	9832 ± 267	17.19 ± 1.02	16.56 ± 2.27	12455 ± 377	10959 ± 536	+18.94 ± 3.61	+4.6 ± 5.13
0.08	10587 ± 136	11144 ± 324	10.15 ± 1.15	5.42 ± 2.75	11749 ± 440	10291 ± 162	+12.19 ± 4.22	+1.75 ± 1.75
0.04	11429 ± 114	11477 ± 105	2.99 ± 0.97	2.58 ± 0.89	11109 ± 577	10742 ± 460	+ 6.09 ± 5.53	+2.58 ± 4.41
control	11782 ± 227				10471 576			

+ = stimulation of uptake

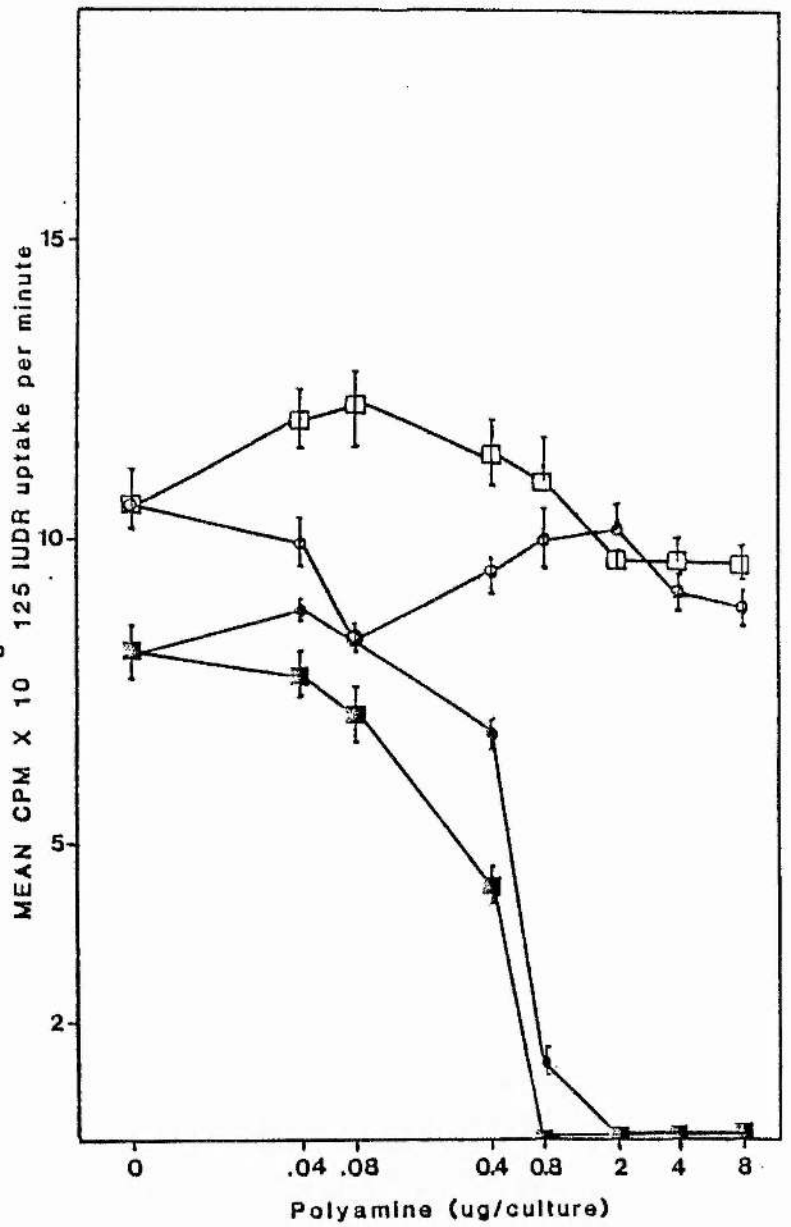


FIGURE 3.1.2B: The uptake of 125IUDR by plasmacytoma cultures, cultured for 4 hours with different concentrations of polyamines per culture. 4/4 incubation sequence.
without FCS: spermidine o——o, spermine □—□
withFCS-HI: spermidine ●——●, spermine ■—■

TABLE 3.1.2B: The effects of spermidine or spermine incubated with FCS in the presence of plasmocytoma cultures for 4 hours on the uptake of 125IUDR by the plasmacytoma culture, (4/4 incubation sequence). The counts are expressed as mean counts per minute, and as percent inhibition. The ± SE is included.
SD = spermidine, SM = spermine.

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	133 ± 11	74 ± 2	98.38 ± 0.13	99.09 ± 0.02	8847 ± 195	9621 ± 284	17.42 ± 1.82	10.19 ± 2.66
4	88 ± 16	107 ± 21	98.92 ± 0.20	98.69 ± 0.025	9226 ± 222	9674 ± 350	13.88 ± 2.08	9.7 ± 3.28
2	105 ± 8	82 ± 9	98.71 ± 0.10	98.99 ± 0.11	10213 ± 271	9702 ± 267	4.66 ± 2.54	9.43 ± 2.50
0.8	1281 ± 151	91 ± 15	84.31 ± 1.86	98.90 ± 0.18	10006 ± 604	11069 ± 572	6.6 ± 5.66	+3.33 ± 5.54
0.4	6797 ± 151	4266 ± 253	16.73 ± 1.86	47.74 ± 3.10	9431 ± 250	11442 ± 579	11.97 ± 2.34	+6.81 ± 5.42
0.08	8388 ± 81	7141 ± 397	+2.77 ± 0.99	12.51 ± 4.88	9402 ± 203	12280 ± 625	12.24 ± 1.89	14.62 ± 5.85
0.04	8874 ± 150	7796 ± 326	+8.72 ± 4.90	4.47 ± 4.0	10075 ± 374	11942 ± 560	5.96 ± 3.50	11.47 ± 5.23
control	8162 ± 478				10713 ± 507			

+ = stimulation of uptake

Thus in the 2 hour preincubation period spermine-FCS generated more isotope uptake inhibition than spermidine-FCS, and consequently while the ID50 for spermine-FCS was 21.53uM, the ID50 for spermidine-FCS could not be determined from the isotope uptake inhibition curves, as the maximum inhibition produced by spermidine was 46.99%.

When the period of polyamine-FCS interaction was increased from 2 to 4 hours, the inhibitory effects of the respective polyamines-FCS, (Figure/Table 3.1.2B) were higher as was evident from the steeper curve gradients of both polyamines between the 0.8 and 8ug/well concentrations, than the respective polyamine-FCS uptake inhibition curves, (Figure/Table 3.1.2A). At concentrations of 0.4 to 8ug/well, (Figure/Table 3.1.2B. PC.4/4 incubation sequence) the respective mean cpm of cultures was significantly different from the cpm of control cultures, ($P<.01$). The inhibitory effects of both polyamines were similar at the 0.8 to 8ug/well concentrations, and were in the 90% region. At 0.4ug/well, spermine was approximately 3 times more effective than spermidine. The ID50 for spermidine (10.99uM) was two folds greater than the ID50 for spermine which was 5.97uM, (Table 3).

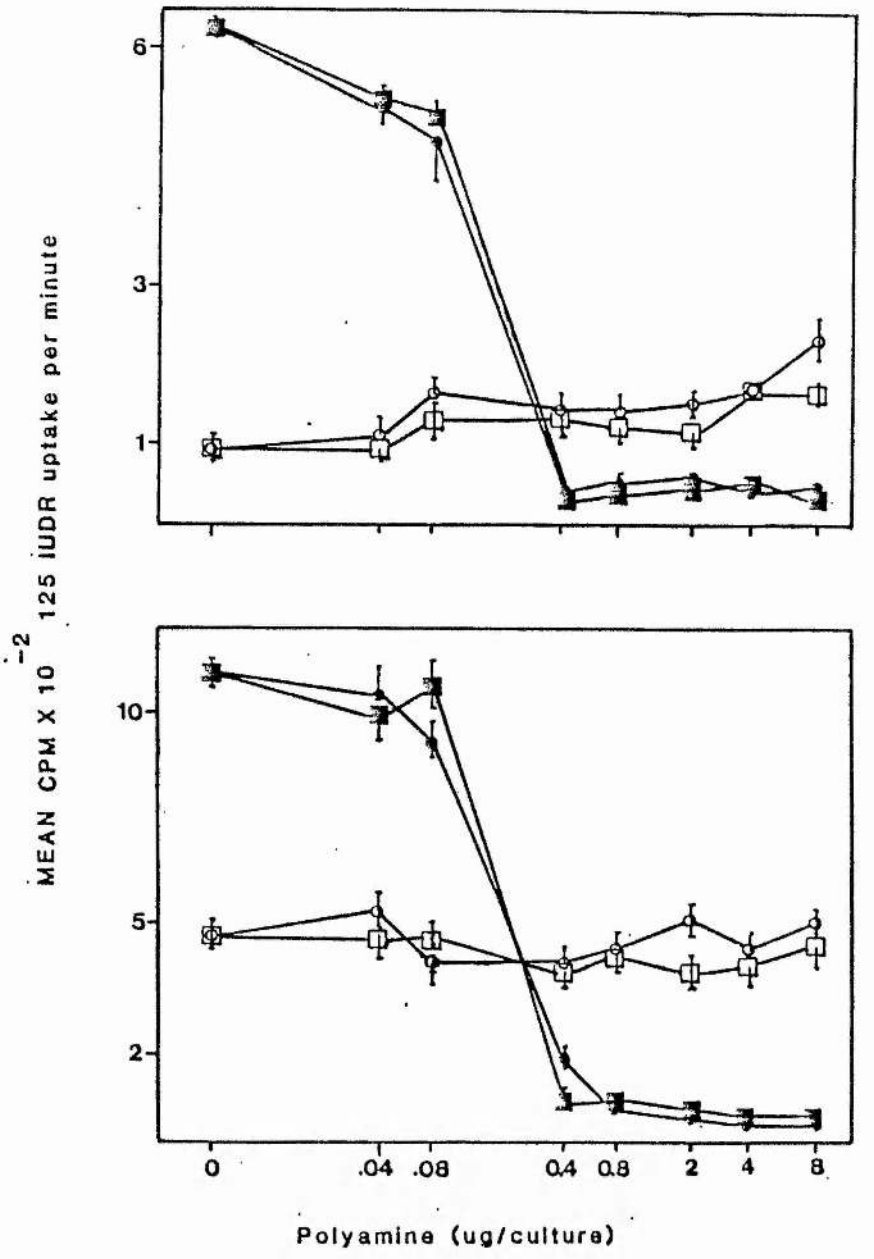
For both sets of cultures, (Figures/Tables 3.1.2A and 2B) cultured with polyamines without FCS, the inhibitory effects of the respective polyamines were marginal, and in some cases the uptake was stimulated. The uptake of isotope was neither linear nor dose dependent, and this contrasts with the inhibitory effects of polyamines which were incubated with FCS.

The inhibitions of isotope uptake by the respective polyamine-FCS incubates on mouse thymocytes cultured at 2/4 and 4/4 incubation sequences, (Figures/Tables 3.1.2C and 2D) were similar between the 0.4 and 0.8ug/well concentrations, and all were in the region of the 90% inhibition value.

The mean cpm of the respective cultures were all significantly ($P<.01$) different from the mean cpm of control cultures. However, with an increase in the incubation period from 2/4 to 4/4, the inhibition of isotope uptake in the 4/4 group was higher at higher dilutions of polyamines (0.04 and 0.08ug/well concentrations), than their counterparts in the 2/4 group. The thymocytes at both incubation periods were more sensitive to the inhibitions of the respective polyamines-FCS, than their plasmacytoma counterparts, (Figures/Tables 3.1.2A and 2B).

The ID50 for the thymocytes at both incubation sequences were similar, (2/4 spermidine-FCS, Spermine-FCS :3.77 and 1.83uM respectively. 4/4 spermidine-FCS, spermine-FCS : 2.98 and 2.35uM respectivley.)

The inhibition of isotope was either marginally inhibited or stimulated, but in either case the change in isotope uptake was neither linear nor dose dependent, when the thymocytes were cultured with the respective polyamines (without FCS). The lack of inhibition of isotope uptake in these cultures, contrasts with the inhibition observed in cultures, containing FCS that reacted with the polyamines to produce the factors that inhibited the uptake of isotope by the respective cultures.



FIGURES: 3.1.2D (above), and 3.1.2C (below):
The uptake of 125IUDR by mouse thymocyte cultures, cultured for 4 hours with different concentrations of polyamines per culture. 2/4 and 4/4 incubation sequence respectively.
without FCS: spermidine o——o, spermine □——□
with FCS-HI: spermidine •——•, spermine ■——■

TABLE 3.1.2C: The effects of spermidine or spermine incubated with FCS in the presence of mouse thymocyte cultures for 2 hours, on the uptake of 125 IUDR by the mouse thymocyte cultures, (2/4 incubation sequence). The counts are expressed as mean counts per minute, and as percent inhibition. The ± SE is included.
SD = spermidine, SM = spermine.

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	44 ± 8	48 ± 6	96.0 ± 0.72	95.56 ± 0.57	519 ± 36	459 ± 29	+6.39 ± 7.42	5.95 ± 6.07
4	36 ± 3	51 ± 8	96.66 ± 0.33	95.35 ± 0.78	454 ± 49	411 ± 36	6.89 ± 10.15	15.74 ± 7.41
2	49 ± 8	69 ± 14	95.52 ± 0.84	93.70 ± 1.43	514 ± 26	392 ± 37	+5.36 ± 5.44	19.68 ± 7.65
0.8	67 ± 16	89 ± 5	93.85 ± 1.54	91.90 ± 0.58	459 ± 32	437 ± 22	5.82 ± 6.70	10.46 ± 4.70
0.4	192 ± 18	92 ± 20	82.53 ± 1.68	91.62 ± 1.87	419 ± 30	399 ± 18	13.98 ± 6.29	18.24 ± 3.76
0.08	941 ± 37	1075 ± 58	14.38 ± 3.44	2.18 ± 5.31	416 ± 34	453 ± 41	14.68 ± 7.04	7.05 ± 8.57
0.04	1049 ± 67	990 ± 54	4.52 ± 6.15	9.91 ± 4.97	538 ± 31	479 ± 48	10.28 ± 6.53	1.77 ± 9.98
control	1099 ± 34				488 14			

TABLE 3.1.20: The effect of spermidine or spermine incubated with FCS in the presence of mouse thymocyte cultures for 4 hours on the uptake of 125IUDR by the mouse thymocyte culture, (4/4 incubation sequence). The counts are expressed as mean counts per minute, and as precent inhibition. The ± SE is included.
SD = spermidine, SM = spermine.

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhbition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	48 ± 8	34 ± 6	92.21 ± 1.53	94.44 ± 1.03	233 ± 30	164 ± 12	+150.53 ± 3.25	+76.34 ± 13.85
4	43 ± 8	54 ± 13	93.08 ± 1.27	91.26 ± 2.61	167 ± 7	165 ± 21	+79.56 ± 7.87	+78.06 ± 22.73
2	56 ± 12	44 ± 9	90.90 ± 2.11	92.82 ± 1.55	152 ± 12	120 ± 15	+64.30 ± 13.69	+29.24 ± 16.96
0.8	49 ± 9	39 ± 6	92.03 ± 1.48	93.73 ± 1.22	141 ± 13	121 ± 14	+52.04 ± 14.81	+30.32 ± 15.18
0.4	40 ± 5	35 ± 5	93.49 ± 0.88	94.38 ± 0.82	145 ± 12	130 ± 19	+56.12 ± 13.84	+70.73 ± 20.60
0.08	482 ± 54	515 ± 20	22.51 ± 8.75	17.18 ± 3.27	165 ± 11	132 ± 20	+77.41 ± 12.21	+42.79 ± 22.15
0.04	520 ± 12	527 ± 13	16.34 ± 1.97	15.18 ± 2.17	105 ± 22	84 ± 10	+13.33 ± 23.88	+ 8.81 ± 10.93
control	622 ± 18				93 ± 16			

As described above, (Figures/Tables 3.1.2A, 2B, 2C and 2D) the effects of the preincubation period of polyamine and FCS (in the presence of cell cultures), on the uptake of isotope by cell cultures was assessed. The results have demonstrated that as the period of incubation was increased, the inhibitory effects produced by the interaction of polyamine with FCS have increased as was evident from the uptake of isotope by plasmacytoma cultures.

The mean cpm of the culture controls (10% FCS) at the 2/4 and 4/4 incubation sequences was 11782 and 8162 respectively, which represented a 30.72% inhibition of isotope uptake by cell cultures for a 2 hour inubation time difference which was significant ($P<.01$). The mean cpm of culture controls without FCS at 2/4 and 4/4 was 10471 and 10713 respectively. This represented a slight stimulation of 125IUDR uptake at 4/4 of 2.25% which was not significant ($P>.05$).

3.3.2: The effects of polyamine-FCS preincubated for 2 or 4 hours at 37 ^{0}C, on the uptake of 125IUDR by cell cultures.

Experimental procedures:

100ul of spermine and spermidine were dispensed into microtitre wells (each polyamine in a separate tissue culture plate) together with 20ul of FCS or without FCS. Several tissue culture plates were prepared, and were incubated at 37 ^{0}C, 5% CO_2, as described earlier. Some plates were incubated for 2 hours and the others for 4 hours.

1:

(100ul of spermine or spermidine + 20ul FCS + 60ul cell suspension + 20ul 125IUDR)

(100ul of spermine or spermidine + 20ulRPMI + 60ul cell suspension + 20ul 125IUDR)

Following the incubation of polyamines for 2 or 4 hours at 37 ^{0}C; separate tissue culture plates received plasmacytoma cells (PC), mouse thymocytes (MTC) or rat thymocytes (RTC), at a concentration of 1 x 10^{5} cells/well; each well received 20ul (0.2uci) 125IUDR and the tissue culture plates were pulsed for 4 hours at 37 ^{0}C, i.e. 2-PC.0/4, 4-PC.0/4, 2-MTC.0/4, 4-MTC.0/4, 2-RTC.0/4, and 4-RTC.0/4 incubation sequences. Each culture was replicated 5 times. The uptake of isotope by cultures was assessed as described previously.

2: In a variation to the above procedure, tissue culture plates received 100ul polyamine + 20ul of FCS or without FCS and were incubated at 37 ^{0}C as above (in number 1) section 3.2. Following the incubation, each plate received 1 x 10^{5} cells/well of one type of cell suspension, of plasmacytoma, normal rat thymocytes or normal mouse thymocytes. The cell cultures were incubated with the polyamines for 4 hours at 37 ^{0}C, followed by the addition of 20ul (0.2uci) of 125IUDR to each well and incubated for a further 4 hours at 37 ^{0}C i.e. 4-PC.4/4, 4-MTC.4/4, 4-RTC.4/4 incubation sequences.

Results:

There was a linear dose response relation between the inhibition of isotope uptake by plasmacytoma cultures, cultured with FCS and the concentration of spermidine and spermine, [(Figures/Tables 3.2.1A and 1B)(2 and 4-PC.0/4 incubation sequences)] In both sets of cultures, the inhibition of isotope uptake between the 2-8ug/well concentrations was significantly different ($P<.01$) from the uptake of isotope by control cultures. In the 4-PC.0/4 incubation sequence, the extra incubation time produced more inhibition on the uptake of isotope as judged from the isotope inhibition curves, (Figure/Table 3.2.1B) which was relatively steeper than their counterpart curves,(Figure/Tables 3.2.1A). The isotope uptake inhibition was relatively greater at the 2 and 8ug/well concentrations, (around 68% for both polyamines at 2ug, and around 80% for both polyamines at 8ug) (Figure/Table 3.2.1.B) than counterpart cultures, (Figure/Table 3.2.1A) in which the inhibition of isotope uptake was relatively lower, (38.9 and 56.8% at 2ug spermidine and spermine respectively, and at 8ug 72.7 and 59.8% respectively).

Further, when the pulsing period was increased from 0/4 to 4/4, (Figure/Table 3.2.1C) there was a marked increase in the inhibition of isotope uptake by both polyamines which was approximately 100%, ($P.<01$ relative to control cultures) at the 2 to 8ug/well concentrations.

Thus the respective ID50 for each polyamine has decreased as the preincubation time or sequence has increased, (Table 3).

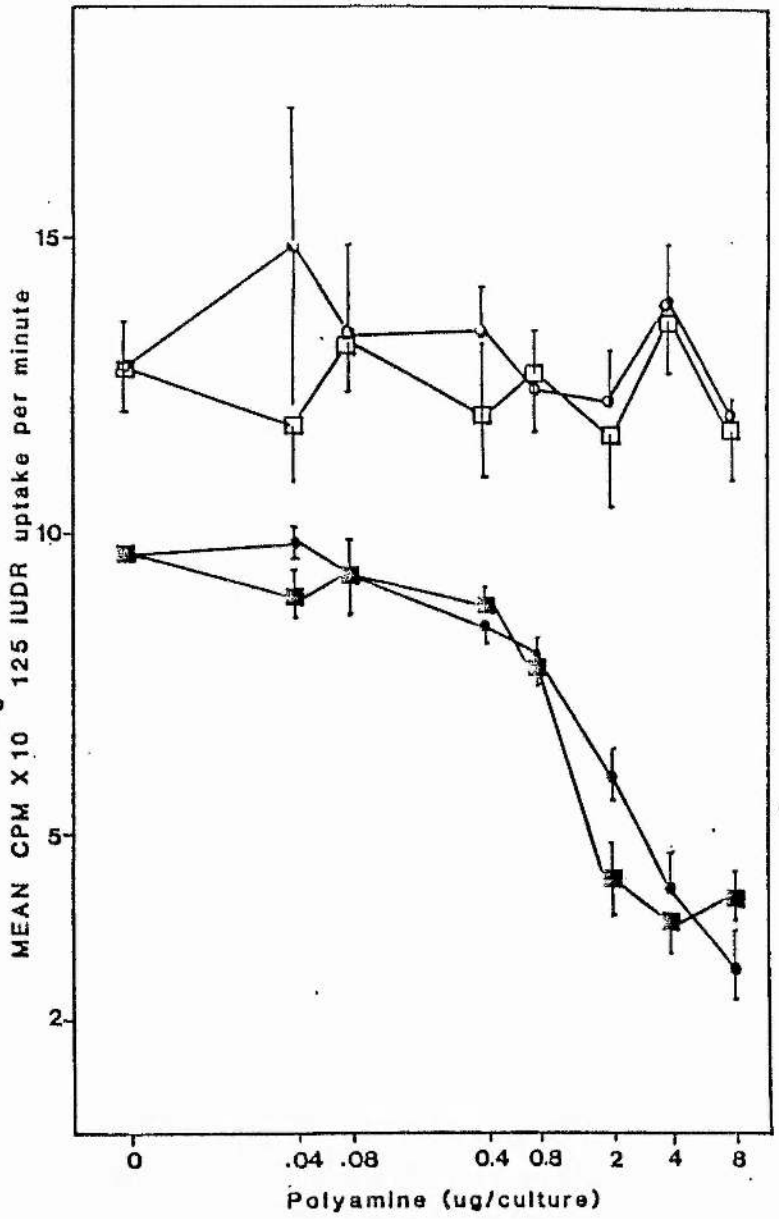


FIGURE 3.2.1A: The uptake of 125IUDR by plasmacytoma cultures, cultured for 4 hours with different concentrations of polyamines per culture. 2-0/4 incubation sequence.
without FCS: spermidine o——o, spermine □——□
with FCS-HI: spermidine •——•, spermine ■——■

TABLE 3.2.1A : The effects of a 2 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by plasmacytoma cultures in a 2-0/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	2693 ± 503	3894 ± 433	72.73 ± 4.07	59.85 ± 7.82	11977 ± 264	11720 ± 764	3.58 ± 2.01	5.65 ± 5.81
4	4004 ± 525	3445 ± 544	58.72 ± 4.25	64.48 ± 4.41	13908 ± 932	13554 ± 799	+11.95 ± 7.09	+9.1 ± 6.07
2	5925 ± 476	4185 ± 635	38.92 ± 3.85	56.86 ± 5.15	12282 ± 824	11681 ± 1244	1.13 ± 6.26	5.96 ± 9.46
0.8	7961 ± 265	7767 ± 322	20.15 ± 2.14	19.93 ± 2.61	12450 ± 765	12671 ± 721	+0.22 ± 5.82	+2.00 ± 5.48
0.4	8451 ± 209	8862 ± 224	12.88 ± 1.69	8.64 ± 1.81	13391 ± 693	11977 ± 1233	+0.77 ± 5.27	3.58 ± 9.38
0.08	9308 ± 619	9364 ± 697	4.05 ± 5.02	3.47 ± 5.65	13334 ± 1568	13347 ± 718	+0.73 ± 11.92	6.63 ± 5.46
0.04	9866 ± 249	8990 ± 345	+1.70 ± 2.01	7.32 ± 2.80	14802 ± 2245	11796 ± 904	+19.15 ± 17.07	5.04 ± 6.87
control	9970 ± 34				12422 ± 742			

+ = stimulation of uptake

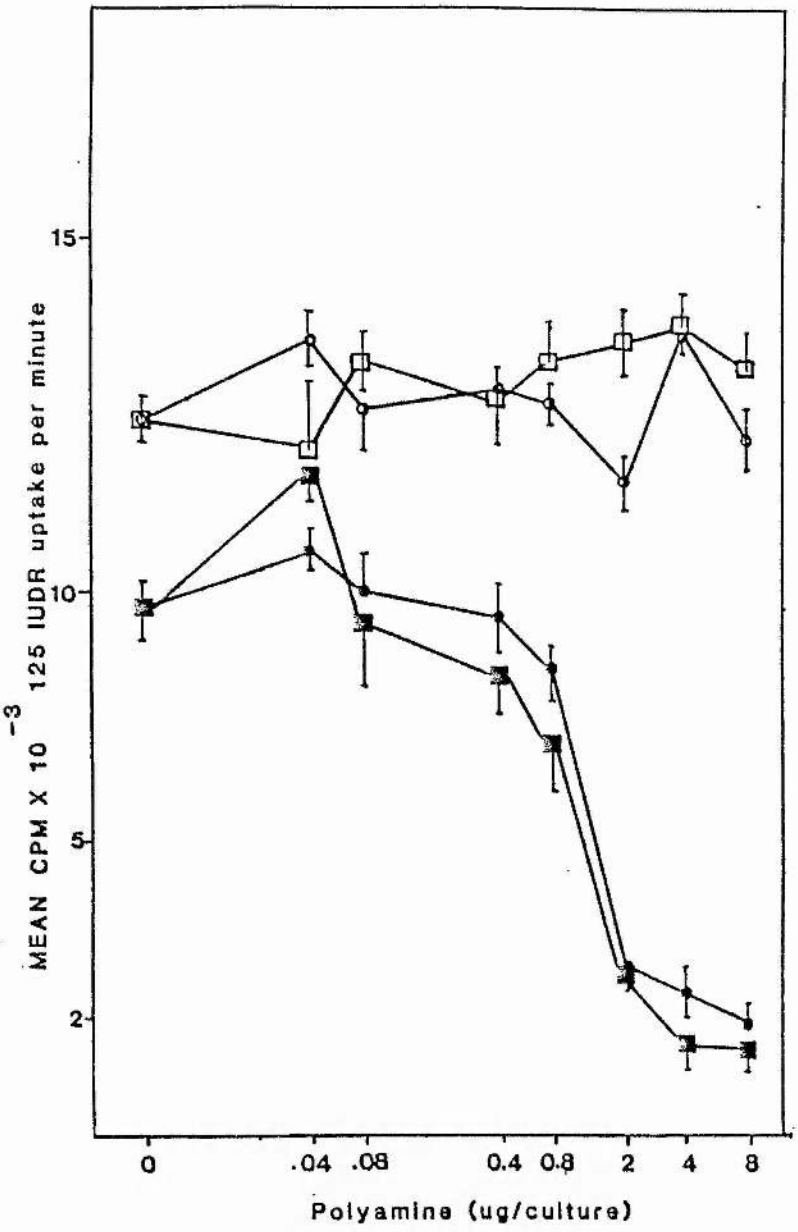


FIGURE 3.2.1B: The uptake of 125IUDR by plasmacytoma cultures, cultured for 4 hours with different concentrations of polyamines per culture. 4-0/4 incubation sequence.
without FCS: spermidine o——o, spermine□—□
with FCS-HI: spermidine •——•, spermine ■—■

TABLE 3.2.1B : The effects of a 4 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by plasmacytoma cultures in a 4-0/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition ± SE		mean cpm ± SE		% inhibition ± SE	
	SD	SM	SD	SM	SD	SM	SD	SM
8	1838 ± 240	1494 ± 282	79.17 ± 2.90	83.07 ± 3.40	11649 ± 485	12807 ± 609	3.22 ± 3.89	+6.39 ± 4.89
4	2391 ± 467	1510 ± 362	72.9 ± 5.67	82.88 ± 4.37	13399 ± 185	13514 ± 422	+11.31 ± 1.49	+12.26 ± 3.39
2	2822 ± 161	2775 ± 261	68.02 ± 1.94	68.55 ± 3.15	10961 ± 447	13306 ± 656	8.94 ± 3.59	+10.53 ± 5.27
0.8	7770 ± 365	6626 ± 754	11.95 ± 4.41	24.91 ± 9.11	12218 ± 1294	12918 ± 619	+1.49 ± 3.59	+7.31 ± 4.97
0.4	8638 ± 487	7762 ± 608	2.11 ± 5.88	12.04 ± 7.34	12549 ± 355	12426 ± 860	+4.24 ± 2.85	+3.22 ± 6.91
0.08	9123 ± 581	8578 ± 1037	+3.37 ± 7.02	2.79 ± 12.53	12162 ± 671	12951 ± 453	+1.03 ± 5.39	+7.58 ± 3.64
0.04	9806 ± 275	11031 ± 368	+11.11 ± 3.32	+24.99 ± 4.44	13368 ± 407	11496 ± 1037	+11.05 ± 3.27	4.49 ± 8.33
control	8825 ± 551				12037 ± 413			

+ = stimulation of uptake

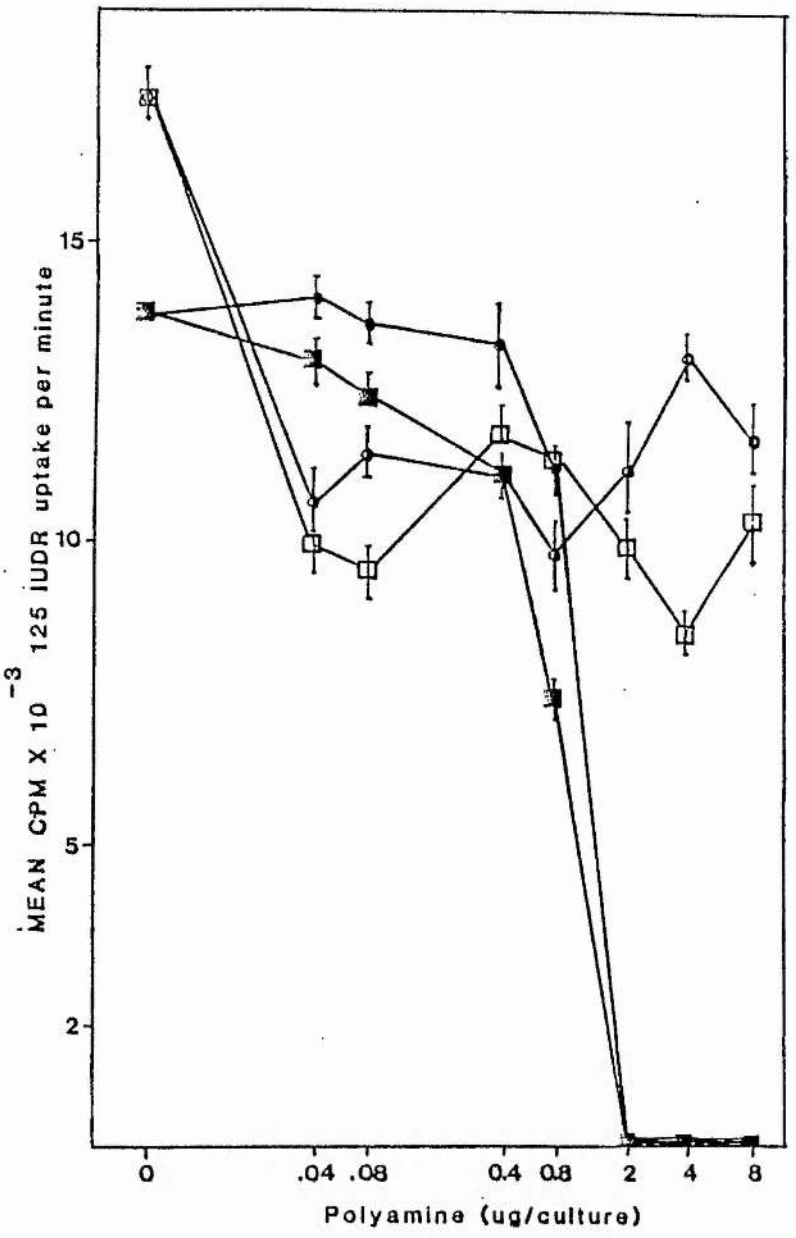


FIGURE 3.2.1C: The uptake of 125IUDR by plasmacytoma cultures, cultured for 4 hours with different concentrations of polyamines per culture. 4-4/4 incubation sequence.
without FCS: spermidine o——o, spermine □—□
with FCS-HI: spermidine •——•, spermine ■—■

TABLE 3.2.1C : The effects of a 4 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by plasmacytoma cultures in a 4-4/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	71 ± 12	55 ± 14	99.48 ± 0.08	99.6 ± 0.10	11722 ± 496	10287 ± 1212	32.87 ± 2.92	41.09 ± 7.13
4	85 ± 15	47 ± 7	99.38 ± 0.01	99.65 ± 0.05	13102 ± 290	8492 ± 308	24.97 ± 1.70	51.37 ± 1.81
2	75 ± 14	65 ± 8	99.45 ± 0.01	99.52 ± 0.05	11215 ± 759	9986 ± 538	35.78 ± 4.46	42.81 ± 3.16
0.8	1176 ± 401	7466 ± 304	18.98 ± 2.90	45.87 ± 2.19	9766 ± 587	11397 ± 144	44.02 ± 3.45	34.74 ± 0.84
0.4	13280 ± 716	11163 ± 343	3.73 ± 5.18	19.07 ± 2.48	11465 ± 793	11746 ± 301	34.35 ± 4.66	32.74 ± 1.76
0.08	13600 ± 280	1244 ± 445	1.41 ± 2.02	9.8 ± 3.22	11523 ± 311	9519 ± 408	34.01 ± 1.83	45.49 ± 2.39
0.04	14026 ± 270	13036 ± 341	+1.67 ± 1.95	5.5 ± 2.47	10680 ± 488	9995 ± 585	38.84 ± 2.87	42.76 ± 3.43
control	13795 ± 137				17464 ± 440			

+ = stimulation of uptake

Spermidine: 2-PC.0/4, 4-PC.0/4 and 4-PC.4/4 = 61.9, 29.06 and 22.78uM respectively.

Spermine: 2-PC.0/4, 4-PC.0/4 and 4-PC.4/4 = 34.46, 18.03 and 12.63uM respectively. In both sets spermine was more inhibitory.

Another feature of this experiment was that the uptake of isotope by cell cultures cultured with the respective polyamines (without FCS) was either slightly inhibited or stimulated, ($P>.05$, relative to control cultures) and the inhibition of isotope uptake was neither linear or dose dependent, (Figures/Tables 3.2.1A and 1B, incubation sequences 2 and 4-PC.0/4 respectively). However, when the incubation sequence was changed to 4/4, (Figure/Tables 3.2.1C) there was a marked inhibition of isotope uptake at all doses of the respective polyamines. The ID50 (polyamine-FCS) for spermidine was 22.78uM and for spermine was 12.63uM which was slightly lower than the respective counterparts described earlier, (Tables 3 and 3.2.1B).

The uptake of 125IUDR by plasmacytoma cultures, cultured with polyamine without FCS, was inhibited at all concentrations for both spermidine and spermine but not in a dose dependent manner. As can be seen in the results (Table 3.2.1C), the inhibition caused by spermidine at all concentrations had a mean of 34.97%, a standard error of 0.378 and a range of inhibition between 24.97% and 44.02.%. Similarly for spermine, the mean inhibition for all cell concentrations had a mean of 41.57%, a standard error of 2.39, and a range of isotope uptake inhibition between 32.74% and 51.37%. The mean cpm of cell cultures in all concentrations of spermidine and spermine, were all significantly different from the mean cpm of culture control ($P<.01$). The inhibitory effects were remarkably different from those described earlier, (Table 3.2.1B).

In effect, upon manipulating culture conditions, by withholding the administration of 125IUDR, and allowing the respective polyamines to interact for a 4-hour period with the respective cultures, followed by the addition of isotope, has resulted in the inhibition of uptake of the isotope by cell cultures. This inhibition of isotope uptake was not evident in the results shown earlier (Table 3.2.1B), where the isotope was added together with the respective polyamine. Similarly, the inhibition of isotope uptake, (Table 3.2.1C) without FCS was more marked than its counterpart (Table 3.1.3B) without FCS.

The inhibition of isotope uptake as assessed on mouse thymocytes, had a similar pattern to that of plasmacytoma cultures, cultured with polyamine-FCS. The inhibition of isotope uptake by polyamines-FCS in the 2-MTC incubation sequence, (Figure/Table 3.2.2A) (spermidine) was in the 60% region and 70% for the other polyamine (spermine), the mean cpm of both polyamines was significantly different ($P<.01$) from the mean cpm of control cultures. These respective inhibitions of isotope uptake have increased to 70 and 90% respectively ($P<.01$, relative to control cultures), when the preincubation period was increased to 4 hours, (Figure/Table 3.2.2B). The inhibition of isotope uptake has further increased, when the period of the incubation was increased to the 4/4 sequence, (Figure/Table 3.2.2C) to produce an inhibition of approximately 100% at the 2-8ug/well concentrations, ($P<.01$, relative to control cultures).

The ID50 for the respective polyamines were as follows:

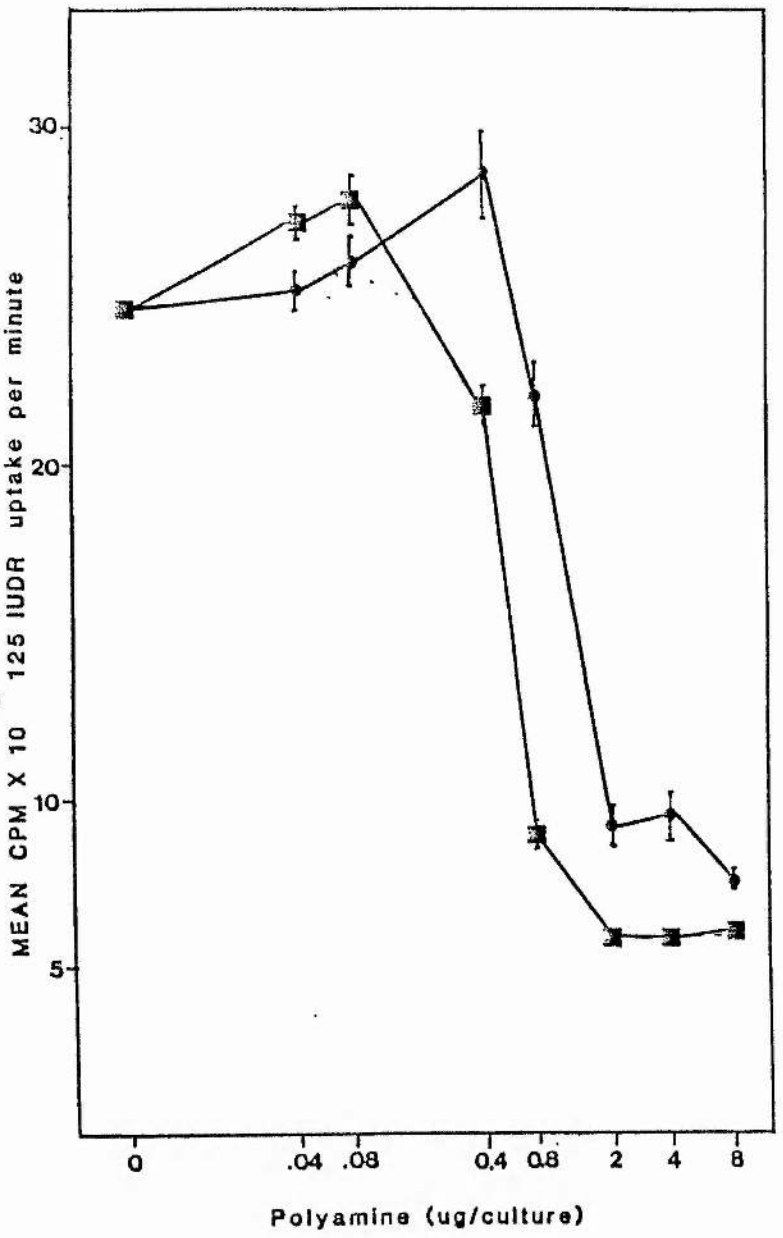


FIGURE 3.2.2A: The uptake of 125IUDR by mouse thymocyte cultures, cultured for 4 hours with different concentrations of polyamines per culture. 2-0/4 incubation sequence.
without FCS: spermidine o——o, spermine □——□
with FCS-HI: spermidine ●——●, spermine ■——■

TABLE 3.2.2A : The effects of a 2 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by mouse thymocyte cultures in a 2-0/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	743 ± 29	594 ± 22	69.77 ± 1.16	75.83 ± 0.88				
4	952 ± 65	573 ± 7	61.26 ± 2.63	76.68 ± 0.28				
2	913 ± 43	585 ± 6	62.85 ± 1.76	76.20 ± 0.26				
0.8	2193 ± 86	881 ± 29	10.78 ± 3.48	64.15 ± 1.16				
0.4	2852 ± 122	2171 ± 48	+16.02 ± 4.96	11.67 ± 1.95				
0.08	2609 ± 78	2773 ± 80	+6.16 ± 3.19	+12.81 ± 3.25				
0.04	2518 ± 49	2707 ± 49	+2.44 ± 2.00	+10.31 ± 1.98				
control	2458 ± 52							

+ = stimulation of uptake

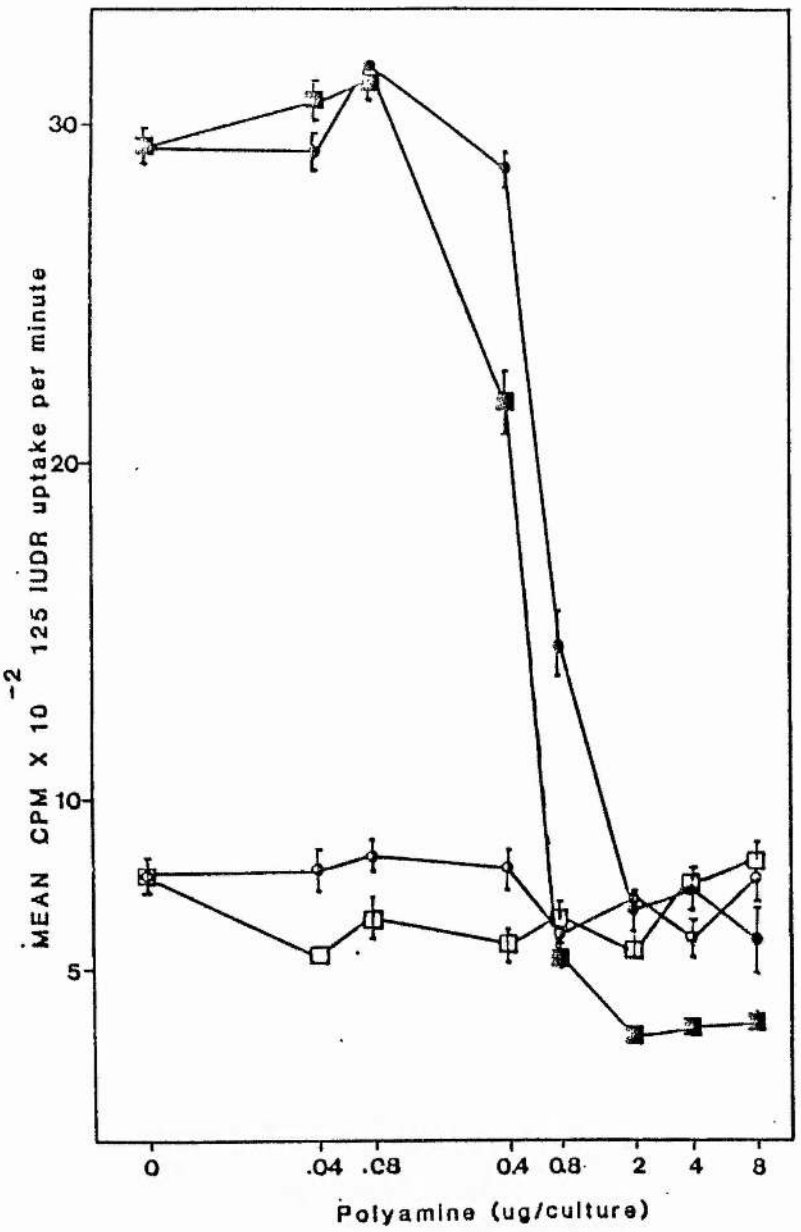


FIGURE 3.2.2B: The uptake of 125IUDR by mouse thymocyte cultures, cultured for 4 hours with different concentrations of polyamines per culture. 4-0/4 incubation sequence.
without FCS: spermidine o——o, spermine □——□
with FCS-HI: spermidine •——•, spermine ■——■

TABLE 3.2.2B : The effects of a 4 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by mouse thymocyte cultures in a 4-0/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	595 ± 91	346 ± 20	79.70 ± 2.37	88.19 ± 0.69	761 ± 69	815 ± 51	2.18 ± 8.83	+4.75 ± 6.54
4	731 ± 66	314 ± 15	75.06 ± 2.27	89.29 ± 0.51	589 ± 47	765 ± 31	24.29 ± 6.06	1.67 ± 3.93
2	670 ± 57	303 ± 22	77.11 ± 1.94	89.66 ± 0.75	683 ± 58	564 ± 31	12.21 ± 7.43	27.5 ± 3.93
0.8	1466 ± 91	539 ± 22	50.00 ± 3.10	81.61 ± 0.74	607 ± 19	641 ± 46	21.97 ± 2.49	17.6 ± 5.87
0.4	2876 ± 69	2170 ± 98	1.90 ± 2.35	25.98 ± 3.36	797 ± 61	577 ± 39	+2.44 ± 7.81	25.83 ± 5.05
0.08	3169 ± 10	3124 ± 62	+8.08 ± 0.35	+6.65 ± 2.12	835 ± 32	650 ± 45	+7.32 ± 4.07	16.43 ± 5.82
0.04	2910 ± 41	3068 ± 59	0.75 ± 1.54	+4.63 ± 2	793 ± 50	555 ± 39	+1.92 ± 6.42	28.66 ± 4.96
control	2932 ± 53				778 ± 33			

+ = stimulation of uptake

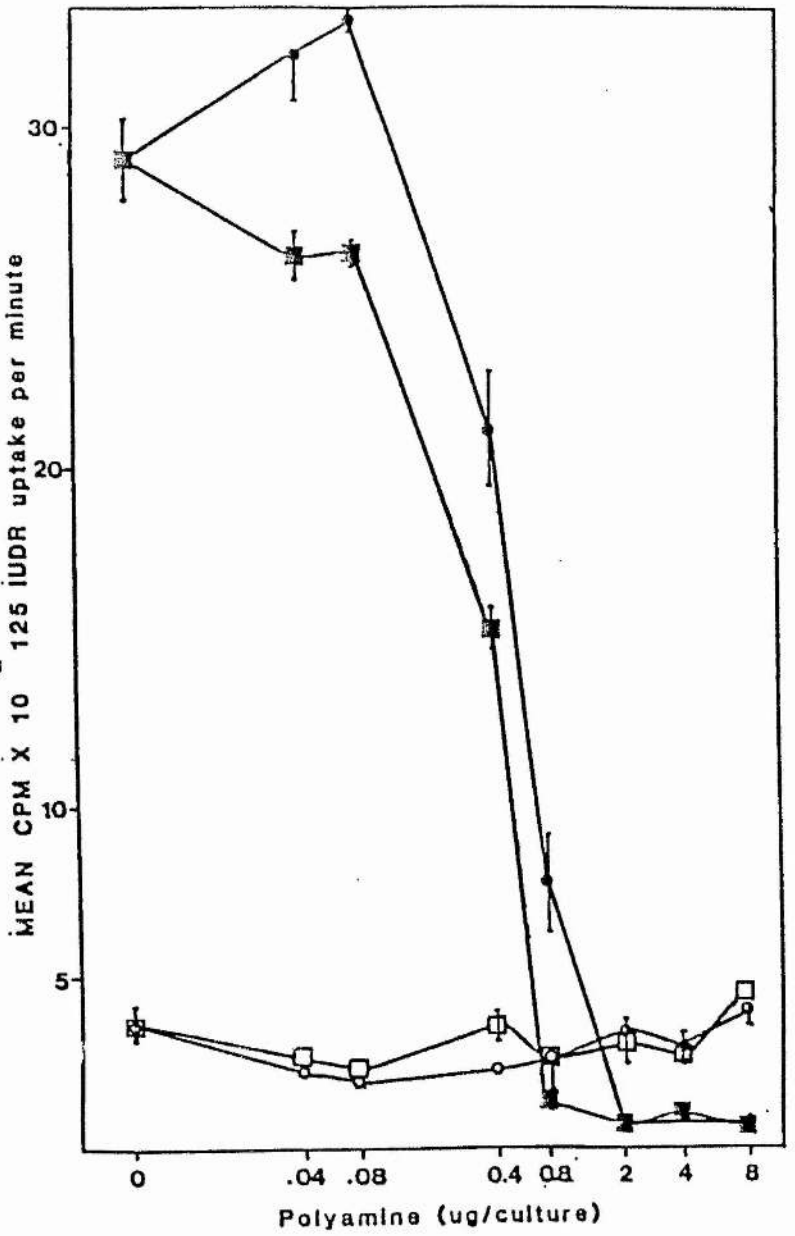


FIGURE 3.2.2C: The uptake of 125IUDR by mouse thymocyte cultures, cultured for 4 hours with different concentrations of polyamines per culture. 4-4/4 incubation sequence.
without FCS: spermidine o——o, spermine □——□
with FCS-HI: spermidine •——•, spermine ■——■

TABLE 3.2.2C : The effects of a 4 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by mouse thymocyte cultures in a 4-4/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	65 ± 15	67 ± 4	97.76 ± 0.49	97.69 ± 0.15	406 ± 38	458 ± 71	+10.62 ± 10.41	+24.1 ± 19.28
4	87 ± 20	108 ± 16	97.00 ± 0.69	96.27 ± 0.54	294 ± 24	298 ± 32	19.89 ± 6.46	18.80 ± 8.61
2	62 ± 14	75 ± 9	97.86 ± 0.48	97.41 ± 0.29	338 ± 46	308 ± 46	7.90 ± 11.45	16.07 ± 12.43
0.8	784 ± 135	134 ± 25	72.98 ± 4.67	95.38 ± 0.89	259 ± 26	257 ± 77	29.42 ± 3.15	29.97 ± 12.6
0.4	2115 ± 165	1529 ± 53	27.11 ± 5.67	47.31 ± 1.82	226 ± 11	346 ± 50	38.41 ± 2.71	5.72 ± 13.70
0.08	3316 ± 42	2630 ± 43	+14.26 ± 1.44	9.37 ± 1.48	204 ± 21	232 ± 11	44.41	36.78 ± 3.03
0.04	3199 ± 145	2627 ± 81	+10.23 ± 4.98	9.47 ± 1.79	223 ± 18	261 ± 15	39.23	28.88 ± 7.41
control	2902 ± 121				367 ± 48			

+ = stimulation of uptake

Spermidine: 2-MTC.0/4, 4-MTC.0/4 and 4-MTC.4/4 = 30.636, 15.71 and 12.56uM rrespectively.

Spermine: 2-MTC.0/4, 4-MTC.0/4 and 4-MTC.4/4 = 9.19, 7.81 and 5.97uM respectively. Spermine was more inhibitory than spermidine, and the thymocytes were more sensitive to the effects of polyamine-FCS than the plasmacytoma cultures, (Figures/Tables 3.2.1A, 1B and 1C.).

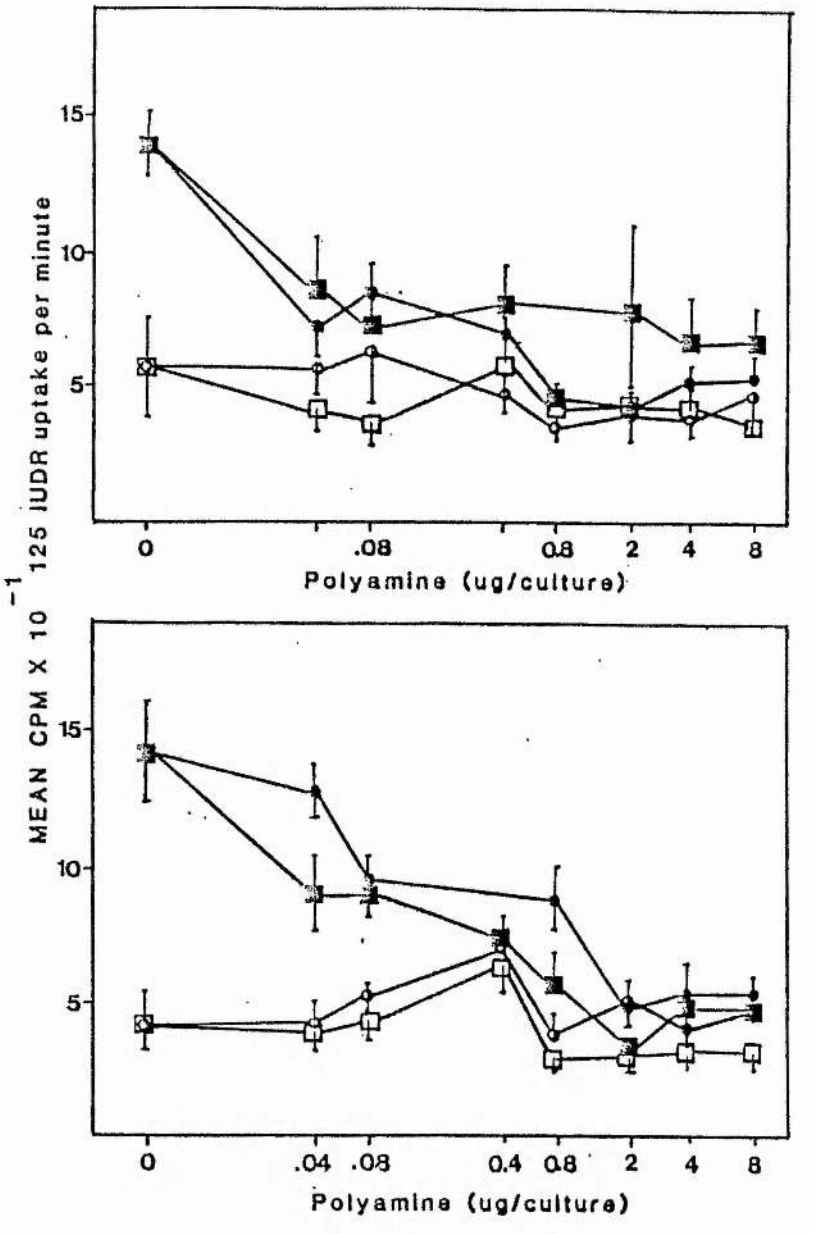
The inhibition of isotope uptake by the respective polyamines and FCS on rat thymocytes was also linear and dose dependent, (Figures/Tables 3.2.3 A, B and C) and the inhibition of isotope uptake (mean cpm of the respective cultures) was significantly different ($P<.01$) from the uptake (mean cpm) of control cultures. However, the extension of the preincubation period from 2 to 4 hours, and the change of the culture incubation sequence from 0/4 to 4/4 did not change the amount of uptake inhibition considerably, (Figures/Tables 3.2.3A, B and C). The respective ID50 for spermidine and spermine, (Table 3).

Spermidine: 2-RTC.0/4, 4-RTC.0/4 and 4-RTC.4/4 = 7.85, 7.85 and 7.12uM respectively.

Spermine: 2-RTC.0/4, 4-RTC.0/4 and 4-RTC.4/4 = 5.74,

1.72 and 1.14uM respectively. Spermine was more inhibitory than spermidine.

The variation in the uptake of isotope by the respective mouse (Figures/Tables 3.2.2A, B and C) and rat (Figures/Tables 3.2.3A, B and C) cultures, cultured with the respective polyamines (without FCS) was neither linear nor dose dependent. As compared to the results of plasmacytoma which were described earlier, (Figure/Table



FIGURES 3.2.3B (above) and 3.2.3A (below):
The uptake of 125IUDR by rat thymocyte cultures, cultured for 4 hours with different concentrations of polyamines per culture. 2-0/4 and 4-0/4 incubation sequence respectively.
without FCS: spermidine o——o, spermine □—□
with FCS-HI: spermidine ●——●, spermine ■—■

TABLE 3.2.3A : The effects of a 2 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by rat thymocyte cultures in a 2-0/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	51 ± 14	46 ± 8	63.82 ± 7.54	67.37 ± 4.31	51 ± 8	30 ± 5	+24.39 ± 14.44	26.82 ± 9.43
4	52 ± 11	47 ± 7	63.12 ± 6.28	66.66 ± 3.73	39 ± 5	30 ± 4	4.87 ± 9.78	26.82 ± 7.61
2	48 ± 9	30 ± 4	66.07 ± 4.77	78.79 ± 2.00	50 ± 7	29 ± 4	21.95 ± 12.72	29.26 ± 7.95
0.8	87 ± 11	55 ± 14	38.51 ± 6.2	61.1 ± 7.67	37 ± 9	28 ± 4	9.7 ± 17.13	31.70 ± 7.37
0.4	71 ± 6	70 ± 16	49.82 ± 3.3	50.53 ± 8.76	22 ± 8	63 ± 11	46.34 ± 20.88	+53.65 ± 20.51
0.08	94 ± 10	88 ± 8	33.56 ± 5.54	37.58 ± 4.53	52 ± 5	42 ± 5	+26.82 ± 9.86	+2.43 ± 9.99
0.04	127 ± 8	91 ± 14	10.34 ± 4.37	35.46 ± 7.91	41 ± 7	39 ± 8	0.00	4.87 ± 14.55
control	141 ± 20				41 ± 11			

+ = stimulation of uptake

TABLE 3.2.3B : The effects of a 4 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by rat thymocyte cultures in a 4-0/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	53 ± 9	65 ± 10	61.59 ± 5.01	52.89 ± 5.69	48 ± 9	37 ± 4	15.78 ± 12.43	35.08 ± 5.75
4	52 ± 8	66 ± 18	62.31 ± 4.75	52.17 ± 10.25	38 ± 5	41 ± 8	33.33 ± 6.54	28.07 ± 10.79
2	42 ± 6	78 ± 33	69.67 ± 3.59	43.68 ± 18.76	40 ± 7	44 ± 3	29.8 ± 9.48	22.80 ± 4.76
0.8	47 ± 6	42 ± 6	66.06 ± 3.29	69.67 ± 3.66	34 ± 4	42 ± 9	40.35 ± 5.30	26.31 ± 12.50
0.4	69 ± 11	80 ± 12	50.0 ± 6.53	42.23 ± 6.71	48 ± 5	58 ± 15	15.78 ± 6.67	+1.75 ± 20.83
0.08	83 ± 12	73 ± 10	39.85 ± 6.75	47.29 ± 5.26	63 ± 19	35 ± 5	+10.5 ± 34.09	38.59 ± 7.10
0.04	70 ± 12	85 ± 20	49.27 ± 6.85	38.62 ± 11.16	57 ± 11	41 ± 7	0.00	28.07 ± 8.98
control	138 ± 11				57 ± 19			

+ = stimulation of uptake

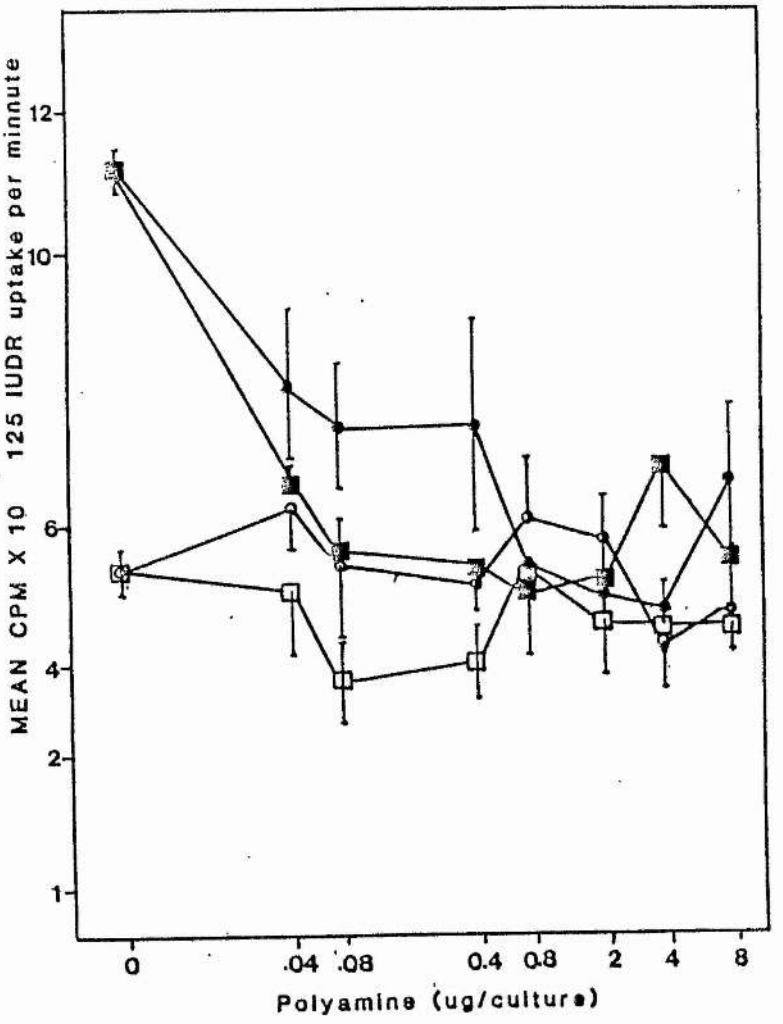


FIGURE 3.2.3C: The uptake of 125IUDR by rat thymocyte cultures, cultured for 4 hours with different concentrations of polyamines per culture. 4-4/4 incubation sequence.
without FCS: spermidine o——o, spermine □—□
with FCS-HI: spermidine •——•, spermine ■—■

TABLE 3.2.3C : The effects of a 4 hour spermidine or spermine - FCS pre-incubate, on the uptake of ^{125}IUDR by rat thymocyte cultures in a 4-4/4 incubation sequence. The counts are expressed as mean counts per minute, and as a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	66 ± 11	55 ± 7	41.07 ± 9.37	50.89 ± 6.49	47 ± 5.5	45 ± 4	11.32 ± 10.62	15.09 ± 8.07
4	47 ± 4	68 ± 8	58.11 ± 4.00	39.28 ± 7.36	43 ± 6	44 ± 6	18.86 ± 13.10	16.98 ± 11.10
2	50 ± 11	52 ± 5	55.44 ± 9.80	53.57 ± 4.83	58 ± 6	46 ± 9	+9.43 ± 11.87	13.20 ± 16.11
0.8	54 ± 6	50 ± 9	51.88 ± 10.35	55.35 ± 7.60	61 ± 9	53 ± 7	+15.09 ± 18.23	0.00
0.4	74 ± 15	53 ± 6	33.92 ± 12.49	52.67 ± 5.65	52 ± 4	40 ± 5	1.88 ± 7.95	24.52 ± 10.32
0.08	74 ± 9	56 ± 5	33.92 ± 7.71	50 ± 4.75	54 ± 11	37 ± 6	+1.88 ± 20.22	30.18 ± 27.91
0.04	80 ± 11	66 ± 7	28.57 ± 9.79	41.07 ± 6.39	62 ± 7	50 ± 8	+16.98 ± 14.13	+5.66 ± 15.14
control	112 ± 3				53 ± 4			

+ = stimulation of uptake

3.2.1C) the results describing the inhibition of isotope uptake by rat thymocytes (Figures/Tables 3.2.3A, B and C) were not as definitive as as that of plasmacytoma, as the mean cpm of the thymocytes control cultures was quite low, (near background levels) to be useful in isotope uptake comparisons with the respective isotope uptake at the respective polyamine dilutions. Similarly, the inhibition of isotope uptake by mouse thymocytes,(Figures/Tables 3.2.2A, B and C) was neither linear nor dose dependent nor was the change in isotope uptake as meaningful as that of the plasmacytoma, as there was not a clear pattern of inhibition at all the respective dilutions of either polyamine.

As described above, (section 3.3.2) the effects of polyamines preincubated with and without FCS on the uptake of isotope by cell cultures were assessed. Two incubation sequences (0/4 and 4/4) were used to assess the contribution of the incubation sequence to the effects of polyamines (with and without FCS) on the uptake of isotope by cell cultures. The results have demonstrated that the increase in incubation sequence from 0/4 to 4/4 increased the inhibition of uptake slightly. The results have also demonstrated (plasmacytoma cultures), that polyamines can be inhibitory without the requirement of FCS, on the uptake of isotope by cell cultures, when the incubation period was changed from 0/4 to 4/4.

3.3.3: The effect of polyamine-FCS added simultaneously with 125IUDR to cell cultures without a preincubation period, on the uptake of isotope by cell cultures.

Experimental procedures:

A plasmacytoma suspension was prepared and dispensed into microtitre wells as described previously. The polyamine and FCS were not incubated as in previous experiments, but were dispensed into tissue culture plates together with the respective cell suspension and 125IUDR and incubated for 4 hours i.e. (0.0/4 incubation sequence). 100 ul of spermine or spermidine together with 20ul FCS were dispensed (each polyamine) into separate microtitre plates, followed by the addition of 1 x 10^5 cells and (0.2uci) 125IUDR to each well and incubated for 4 hours at 37 ^{0}C, 5% CO_2.

[100ul polyamine +20ul FCS]+60ul suspension+20ul 125IUDR

[100ul polyamine +20ul RPMI]+60ul cell suspension+20ul 125IUDR

The cell cultures used for this experiment were plasmacytoma and rat thymocytes. The incubation sequence was as follows: O-PC. 0/4, O-RTC 0/4. Each culture was replicated 5 times. The uptake of isotope by cultures was assessed as described previously.

Results:

The inhibition of isotope uptake by the respective polyamine-FCS mixtures on plasmacytoma cultures [(Figure/Table 3.3.1A)(O-PC.0/4 incubation sequence)], was linear and dose dependent. The uptake of isotope at 0.8 to 8ug/well concentrations was significantly different from the uptake of isotope by control

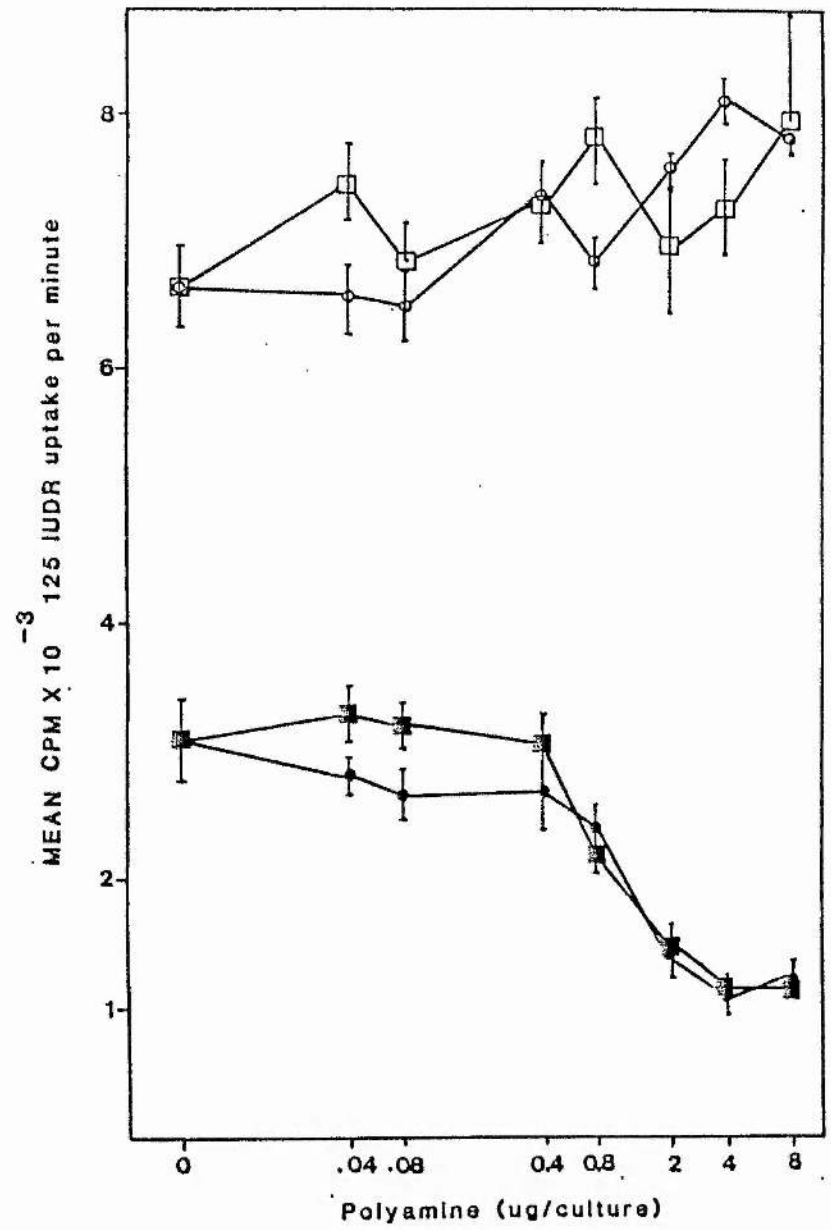


FIGURE 3.3.1A: The uptake of 125 IUDR by plasmacytoma cultures, cultured for 4 hours with different concentrations of polyamines per culture. 0-0/4 incubation sequence
without FCS: spermidine o——o, spermine □—□
with FCS-HI: spermidine •——•, spermine ■——■

TABLE 3.3.1A : The effects of the simultaneous addition of spermidine or spermine and FCS and ^{125}IUDR to plasmacytoma cultures in a 0-0/4 incubation sequence on the uptake of ^{125}IUDR by plasmacytoma. The counts are expressed as mean counts per minute, and a percent inhibition. The ± SE is included.

SD = spermidine SM = spermine

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	1216 ± 139	1175 ± 97	60.9 ± 4.4	62.21 ± 3.13	7790 ± 74	7939 ± 847	+14.28 ± 1.40	+16.46 ± 12.45
4	1107 ± 133	1153 ± 60	60.4 ± 4.31	62.91 ± 1.95	8099 ± 176	7254 ± 414	+18.81 ± 2.60	+6.42 ± 6.09
2	1434 ± 190	1486 ± 150	52.88 ± 6.13	52.20 ± 4.84	7559 ± 125	6943 ± 560	+10.89 ± 1.84	+1.85 ± 8.24
0.8	2415 ± 173	2186 ± 92	22.34 ± 5.57	29.70 ± 2.98	6819 ± 215	7784 ± 510	+.03 ± 3.16	+14.19 ± 7.5
0.4	2701 ± 282	3064 ± 209	13.16 ± 9.10	1.47 ± 6.75	7339 ± 274	7280 ± 315	+7.65 ± 5.19	+6.79 ± 1.74
0.08	2686 ± 238	3231 ± 137	13.63 ± 7.66	+3.89 ± 4.4	6466 ± 269	6854 ± 302	5.13 ± 3.96	+0.5 ± 4.41
0.04	2832 ± 96	3336 ± 204	8.94 ± 3.11	+7.27 ± 6.57	6562 ± 267	7423 ± 287	3.73 ± 3.96	+8.9 ± 4.23
control	3110 ± 350				6817 ± 115			

+ = stimulation of uptake

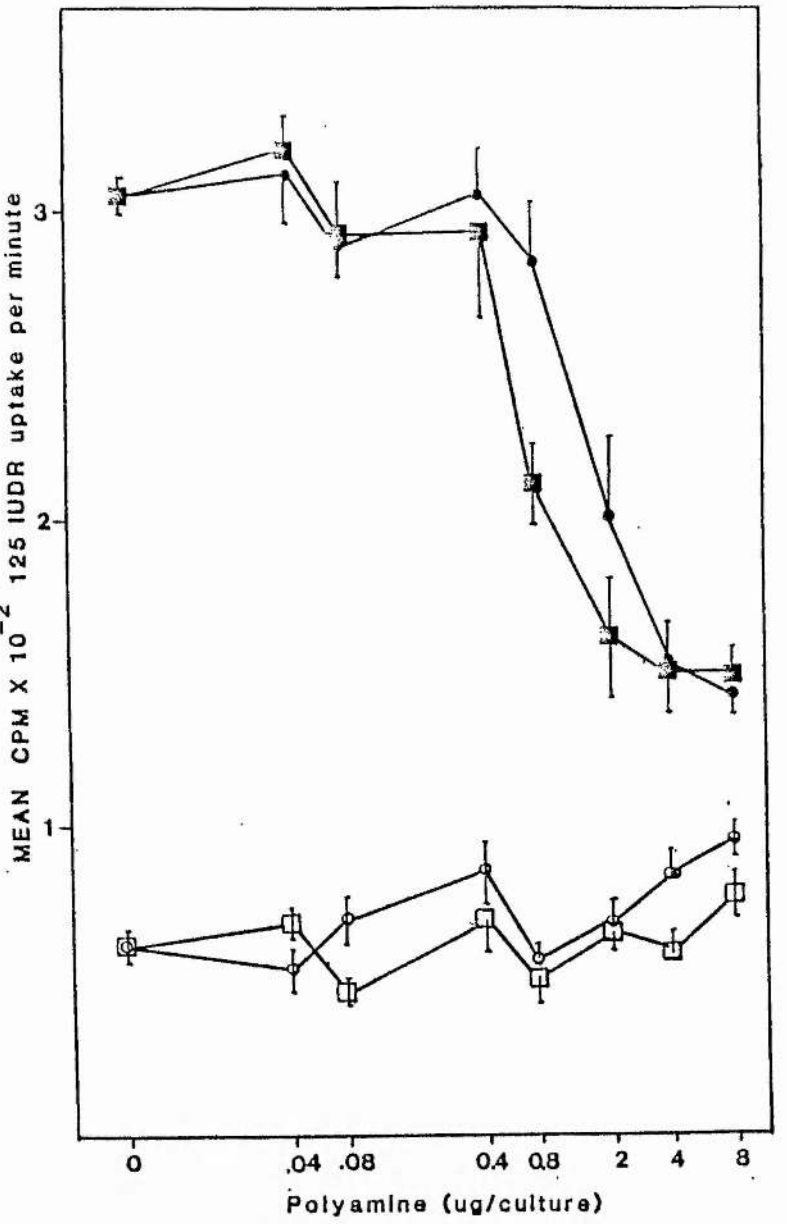


FIGURE 3.3.1B: The uptake of 125 IUDR by rat thymocyte cultures, cultured for 4 hours with different concentrations of polyamines per culture. 0-0/4 incubation sequence
without FCS: spermidine o——o, spermine □—□
with FCS-HI: spermidine •——•, spermine ■——■

TABLE 3.3.1B : The effects of the simultaneous addition of spermidine or spermine and FCS and ^{125}IUDR to rat thymocyte cultures in a (0-0/4 incubation sequence) on the uptake of ^{125}IUDR by rat thymocyte cultures. The counts are expressed as mean counts per minute, and as percent inhibition. The ± SE is included.

polyamine conc. µg/well	with 10% FCS				without FCS			
	mean cpm ± SE		% inhibition		mean cpm ± SE		% inhibition	
	SD	SM	SD	SM	SD	SM	SD	SM
8	143 ± 5	150 ± 7	53.11 ± 1.59	50.81 ± 2.43	95 ± 3	77 ± 7	+53.22 ± 5.31	+24.19 ± 11.76
4	154 ± 12	150 ± 14	49.50 ± 4.27	50.81 ± 4.80	74 ± 7	58 ± 6	+19.35 ± 12.40	+6.45 ± 10.79
2	200 ± 27	161 ± 20	34.42 ± 9.05	47.21 ± 6.68	68 ± 7	65 ± 4	+9.67 ± 11.52	+4.83 ± 6.65
0.8	283 ± 23	212 ± 13	7.21 ± 7.81	30.49 ± 4.54	57 ± 4	50 ± 7	8.07 ± 6.72	19.35 ± 12.22
0.4	305 ± 10	293 ± 29	0.00	+3.93 ± 9.81	84 ± 10	69 ± 7	+35.48 ± 16.92	+11.29 ± 11.83
0.08	290 ± 13	292 ± 16	4.91 ± 4.36	4.26 ± 5.47	71 ± 5	46 ± 4	+14.51 ± 9.53	25.80 ± 6.69
0.04	311 ± 14	320 ± 12	+1.96 ± 4.77	+4.92 ± 4.15	54 ± 6	68 ± 4	12.90 ± 9.99	+9.67 ± 6.73
control	305 ± 5					62 ± 3		

+ = stimulation of uptake

cultures (P<.01). The uptake of isotope ranged from 20 to 60% for each of the respective polyamines.

Similarly, the inhibition of isotope uptake by the respective polyamine-FCS mixtures on rat thymocytes [(Figure/Table 3.3.1B)(0-RTC.0/4 incubation sequence)], was linear and dose dependent. The uptake of isotope at the 2 to 8ug/well concentrations was significantly different from the uptake (P<.01) of control cultures. The uptake ranged from 30 to 50% for spermidine and was around 50% for the three respective dilutions of spermine.

It can be seen (Table 3), that the ID50 for plasmacytoma was lower than the ID50 of rat thymocytes. The relative change in the respective sensitivities of the respective cultures was related to the change in the variables of culture incubation, (i.e. period of incubation of polyamines with FCS, the timing of the addition of polyamines to cultures with or without isotope and the length of the incubation period), as the timing of the addition of polyamines (which were not preincubated), FCS and isotope were all set at time zero,(same experimental time).

It was evident in previous results (Figures/Tables 3.1.1A and 1C) (Table 3) (Figure/Table 3.2.1B and 3B), that the ID50 for the thymocytes was lower than plasmacytoma. Thus the amount of isotope uptake by polyamine-FCS mixtures on the respective cell types was related to the timing of application of the inhibitory substance and isotope to the cell cultures.

The ID50 for the respective cultures, (Table 3) were as follows:

Plasmacytoma: 34.56 and 24.12uM respectively for spermidine and spermine.

Rat thymocytes: 78.55 and 57.43uM respectively for spermidine and spermine.

As described above (section 3.3.3) the relative sensitivities of cell cultures (normal and transformed) to the inhibitory effects of polyamine-FCS mixtures, were assessed. It was demonstrated in previous results (sections 3.3.1 and 3.3.2) that the thymocytes were more sensitive than the plasmacytoma to the inhibitory effects of polyamine-FCS. The results described above have demonstrated that the sensitivity of cell cultures to the inhibitory effects of polyamine-FCS was related to the culture incubation conditions.

3.3.4: Polyamine reversibility: The effects of exposing cell cultures to polyamine-FCS for a short period, on the uptake of 125IUDR by the same cell cultures without the presence of polyamine-FCS.

Experimental procedures:

A suspension of plasmacytoma cells was prepared as described previously and was divided into 3 sets. The first set contained 39.27 uM of spermidine in 10% FCS, the second contained 28.71 uM spermine in 10% FCS, while the third group contained 10% FCS without polyamines. Five replicate sterile tubes for lymphocyte culture (NUNC., U.K.) were prepared for each set, each tube containing ($2x10^6$ cells/ml) with the respective polyamines for sets 1 and 2. The polyamine-FCS cell culture and isotope pulsing was similar to the procedure for the experiment which was described in

Table 3.2.1C and as described in materials and methods, (i.e. 4-PC.4/4 incubation sequence). All cultures were incubated for 4 hours at 37 °C, as described earlier. Following the incubation, the tubes were centrifuged for 5 minutes (1.5×10^3 RPM), after which the supernate was discarded. The cell pellet in each tube was resuspended in 900ul of warm 10% FCS-RPMI. Each culture received 100ul of 125IUDR (1uci) and all cultures were incubated for 4 hours. Following the incubation, the uptake of isotope was assessed as described previously.

Results:

The respective mean cpm of cell cultures, cultured with spermidine or spermine, was lower than the mean cpm of 10% FCS control cultures by 18.51% and 2.75% respectively, (Table 3.4). The differences between the respective means were significant in an Anovar; (F.ratio = 4.574, $P<.05$). However the differences among the means were not significant as assessed by Dunnett's comparison of means procedure.

The results have demonstrated that the inhibitory effects of polyamine-FCS on cell cultures were not cytotoxic but were reversible and without a lasting effect either on the cell membrane or on the process of DNA synthesis.

TABLE 3.4 : The reversible effects of polyamine-FCS on the uptake of ^{125}IUDR by plasmacytoma cultures. The values are expressed as mean cpm with ± SE values included.

Polyamine concentration	Spermidine/FCS	Spermine/FCS mean cpm ± SE	10% FCS control without polyamine
28.71μM		158111 ± 5981	162588 ± 5526
39.27μM	132482 ± 10377		

TABLE 3.5 : The cell viabilities of plasmacytomas and mouse thymocytes cultured in polyamine-FCS.

Percent change in cell viability					
	Plasmacytoma			Thymocytes	
Time/hours	SD	SM	C	SD	SM
4	+6.94 ± 7.83	+1.38 ± 12.87	100%	-2.66 ± 10.07	-9.77 ± 8.24
6	+10.32 ± 6.80	+5.8 ± 8.24	100%	-9.17 ± 3.182	-17.88 ± 6.62
8	+9.23 ± 18.21	-12.3 ± 14.85	100%	-1.76 ± 10.21	-0.58 ± 6.63
				+1.03 ± 2.81*	+0.98% ± 1.51*

SD = spermidine

SM = spermine

C = control cultures without polyamine (100% without change)

* = rat thymocytes (8 hours)

TABLE 3 : The dose of polyamine in micromoles, which is required for 50% inhibition (ID50) of ^{125}IUDR uptake by polyamine-FCS on the respective cell cultures.*

Section 3.1	Spermidine	Spermine	Table Figure
PC. 0/4 =	16.967	20.103	3.1.1A
M.aden. 0/4 =	16.810	7.581	3.1.1B
RTC. 0/4 =	5.027	3.676	3.1.1C
MTC. 0/4 =	6.284	2.871	3.1.1D
PC. 2/4 =	-	21.539	3.1.2A
PC. 4/4 =	10.997	5.973	3.1.2B
MTC. 2/4 =	3.770	1.838	3.1.2C
MTC. 4/4 =	2.985	2.354	3.1.2D
Section 3.2			
2-PC. 0/4 =	61.900	34.462	3.2.1A
4-PC. 0/4 =	29.065	18.035	3.2.1B
4-PC. 4/4 =	22.780	12.636	3.2.1C
2-MTC. 0/4 =	30.636	9.190	3.2.2A
4-MTC. 0/4 =	15.710	7.811	3.2.2B
4-MTC. 4/4 =	12.568	5.973	3.2.2C
2-RTC. 0/4 =	7.855	5.743	3.2.3A
4-RTC. 0/4 =	7.855	1.723	3.2.3B
4-RTC. 4/4 =	17.124	1.148	3.2.3C
Section 3.3			
0-PC. 0/4 =	34.563	24.124	3.3.1A
0-RTC. 0/4 =	78.554	57.438	3.3.1B

PC = Plasmacytoma M.aden = Mammary adenocarcinoma

RTC = Rat thymocytes MTC = Mouse thymocytes

0/4 = ^{125}IUDR added to cultures at zero time (with polyamine) and incubated for 4 hours.

2/4 = Polyamine incubated for 2 hours with culture. Then ^{125}IUDR was added and culture incubated for 4 hours.

4/4 = Polyamine incubated for 4 hours with culture. Then ^{125}IUDR was added and culture incubated for 4 hours.

(2 or 4) PC or MTC or RTC (0/4 or 4/4) = Polyamine incubated with FCS for either 2 or 4 hours at 37°C, then added to cell cultures together with isotope (i.e.: 0/4) or added to culture for 4 hours (i.e.: 4/4), after which the cultures are pulsed with ^{125}IUDR for 4 hours.

* see text for details

3.4. THE GROWTH KINETICS OF PLASMACYTOMA IN BALB/C MICE.

3.4.1: The growth rate of plasmacytoma in vivo in Balb/c mice:

Experimental procedures:

Thirty normal Balb/c mice (around 8 weeks of age) were divided into 3 groups of 10 mice each. A plasmacytoma cell suspension was prepared as described previously in RPMI 1640 without FCS and was diluted into 3 different concentrations of 1×10^7, 5×10^6 and 1×10^6 cells per ml. Each mouse received 0.1 ml of a cell suspension as described previously. The dose for the first group of mice was 1×10^6 cells/mouse. The dose for the second group was 5×10^5 cells/mouse, while the dose for the third group of mice was 1×10^5 cells/mouse. The mice were observed daily to note tumour growth, and when the tumours were palpable at the earliest size (tumour size group 3), the tumour size of each mouse was recorded , thereafter every 48 hours, until reaching the final volume (tumour size group 7).

Results:

The results (Figure/Table 4.1) for the growth rates of the respective cell implants into mice, were similar and were not significantly different among each other (m1 and m2- $P>0.5$), (m1 and m3- $P>0.5$), and (m2 and m3- $P>0.5$) although the tumour doubling time was slightly shorter at a higher cell dose at approximately 2 hours difference for each cell dose. Vo., and Vmax., were reached earlier for the relatively higher cell dose at 2 days respectively.

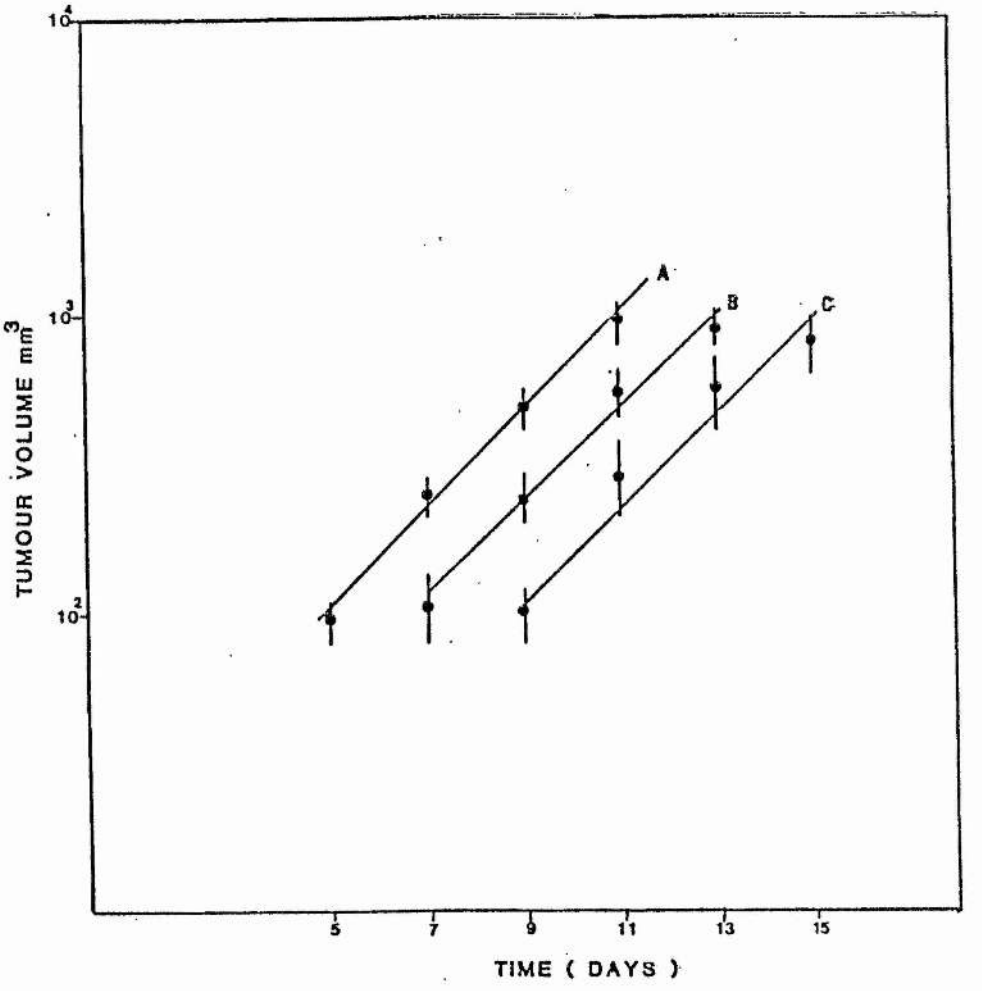


FIGURE 4.1: The growth curves of plasmacytoma of different initial cell inocula in Balb/c mice.
A: 1 x 10^6cells/mouse; B: 5 x 10^5cells/mouse; C: 1 x 10^5cells/mouse.

TABLE 4.1 : The growth rates and tumour doubling times of plasmacytoma in yivo in Balb/c mice innoculated at different cell concentrations of 1×10^6, 5×10^5 and 1×10^5 cells per mouse.

Cell dose/mouse		M ± SE	Td (hr)	Vo, Vo.t	Vmax, Vmax.t	Δt
A	1×10^6	0.378 ± 0.017	44.009	~3.5+, day 5	7.5~+, day 11	6 days
B	5×10^5	0.357 ± 0.019	46.59	~3.5+, day 7	7.5~+, day 13	6 days
C	1×10^5	0.341 ± 0.036	48.78	~3.5+, day 9	~7.5, day 15	6 days

Vo = initial volume (first reading)
Vo.t = time at which tumour reached Vo
Vmax = final volume (last reading)
Vmax.t = time at which tumour reached Vmax
Δt = period of tumour observation and measurements
M = slope KG (growth rate)
Td = tumour doubling time

Therefore the 5 x 10^5 cell dose was chosen as the standard cell dose for further experiments.

As described above, the relation between the growth rate of tumours and cell dose injected into mice was assessed. The results have demonstrated that the growth rate and hence the doubling time of the tumour was independent from the cell dose, i.e. a higher cell dose does not produce a tumour with a faster growth rate (shorter doubling time) relative to the growth rate of tumours which grew from smaller cell implants.

3.4.2.1: The growth rates of plasmacytoma in normal Balb/c mice and congenitally athymic "nude" mice with Balb/c background:

Experimental procedures:

A plasmacytoma suspension was prepared as described earlier and adjusted to a concentration of 5 x 10^6 cells/ml in RPMI 1640 without FCS. Two groups of mice (10 per group), the first, was normal Balb/c and the second was the congenitally athymic "nude" mice with Balb/c background, were injected with 0.1 ml (5 x 10^5 cells) of the plasmacytoma suspension per each mouse as described previously . The mice were left for a period of time, until the tumours were palpable at size 3.5, and thereafter the tumour size was recorded every 48 hours as described previously.

Results:

The growth rate which is the slope (m or KG) was slightly faster (36% faster) in the "nude" mice compared to control Balb/c, (Figure/Table 4.2.1) but the difference was not ($P>0.05$)

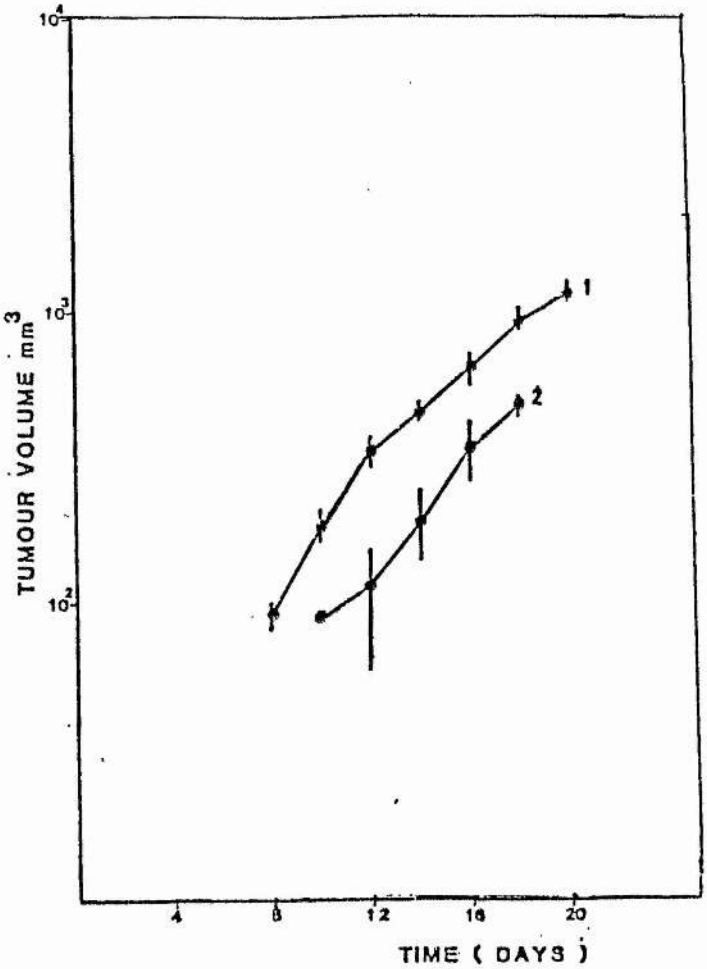


FIGURE 4.2.1:The growth curves of plasmacytoma in Balb/c mice and in congenitally athymic Balb/c mice.

1: Balb/c 2: athymic Balb/c

TABLE 4.2.1 : The growth rate and related growth kinetic parameters of plasmacytoma growing in Balb/c normal mice and in congenitally athymic mice with Balb/c background.

Kinetic Parameter	Balb/c normals	Athymic mice (nu/nu)	t
Int.	36.00	6.77	
M ± SE	0.175 ± 0.0235	0.239 ± 0.0241	t = 1.904, 0.1 > P > 0.5
r	0.9927	0.9945	
Td (hrs)	95.06 ± 14.842	69.60 ± 3.585	t = 1.668, 0.1 > P > 0.5
Lp (days)	5.82 ± 0.786	11.24 ± 1.136	t = 3.924, P < .01

Int. = intercept
M = slope (growth rate = KG) with ± standard error included
r = correlation coefficient
Td = tumour doubling time with ± standard error included
Lp = latency period for tumour to reach $100mm^3$ with ± standard error
SE = standard error
t = t-test comparison

significant. The corresponding tumour doubling time (Td) was higher for Balb/c than for "nude" mice by 26.7%, but similarly the difference was not significant ($P>0.05$). However, the latency period which was the time required for the tumour to reach a specified volume of (100 mm) was about twice as long for "nude" mice, relative to normal Balb/c mice, which was a significant difference, ($P<.01$)

As described above,(section 3.4.2.1) the kinetics of tumour growth in two different immunological environments (nude and normal mice) were assessed. The results have demonstrated that the latency period (the period for the tumour to reach a specified volume after the implantation of tumour cells into mice) was quite different between the two environments. The tumour took more time to reach the pre-set tumour volume in "nude" than in normal mice. However, the overall growth rate in the "nude" mice was relatively faster than in normal mice, but the difference was not significant.

3.4.2.2.1: The mean Birth rate and related growth kinetic parameters of plasmacytoma growing in Balb/c mice at tumour sizes (5, 6 and 8), and in congenitally athymic mice with Balb/c background:

Experimental procedures:

A plasmacytoma suspension was prepared and was adjusted to a concentration of (5×10^6 per ml). Seventy Balb/c mice and 18 athymic "nude" were injected in the inguinal region with an inoculum of 5×10^5 cells per mouse as described previously. When the tumours reached a palpable size, tumour growth was measured as described previously. Three tumour sizes (tumour size, 5,6 and 8)

were chosen for the stathmokinetic experiment, and when the tumours reached these sizes, each mouse was injected with 0.25ml of vincristine sulphate solution as described previously. Following the administration of vincristine, the tumours were removed, at the following time intervals of 0, 0.5;1;1.5;2;2.5;3;3.5; and 4 hours. Each time interval was duplicated, thus, for example, for tumour size 5, 18 tumours were used; 2 mice per each time interval. The tumours were processed for histological studies as has been described previously. The procedure for the estimation of mean birth rate and related kinetic parameters has been described in materials and methods.

Results:

The mean birth rate (KB) [unweighted regression analysis] dropped progressively as the tumour size increased (Table 4.2.2.1), with a corresponding increase in (Tca) the mean apparent cell cycle time. The increase in tumour volume from size 5 to 6 was accompanied by a reduction in the mean birth rate of approximately 12% (0.02>P>.01), and as the tumour volume increased to size 8, the mean birth decreased by 15% (0.01>P>.001). Thus the change in the mean birth rate between tumour sizes 5-6 and 5-8 was significant, while the difference in the mean birth rate between tumour sizes 6 and 8 which was 3.5%, was not significant (P>.05). The Tca at tumour size 6 increased by approximately 5 hours, relative to tumour size 5 which was an increase of approximately 13%, which was a significant difference (0.02>P>0.01). The difference in the Tca between tumour sizes 5 and 8 was 6.58 hours which was significant (P.<.01), which was an increase of approximately 18% at size 8 relative to size 5. The increase in Tca from size 6 to size 8 was

TABLE 4.2 2-1 : Metaphase collection function.
A. Unweighted regression analysis : Birth rates and related parameters at tumour size 5, 6 and 8 for Balb/c mice and size 6 for nude mice with Balb/c background.

	5	6	8	nu/nu 6	t
Int.	0.002454	0.003019	0.003393	0.00378	KB5 vs KB6
± SE	0.000738	0.000635	0.000536	0.00104	t = 2.344, 0.02 > P > .01, S
KB	0.008038	0.007084	0.006836	0.009418	KB5 vs KB8
± SE	0.000308	0.000266	0.000224	0.000959	t = 3.156, 0.01 > P > .001, S
CPR	8.038	7.084	6.836	9.418	KB6 vs KB8
Tca	37.451	42.494	44.036	31.960	t = 0.713, P > 05, NS
± SE	1.4336	1.597	1.4401	3.254	KB6 vs KB6 Nude
tm	0.3053	0.4262	0.4963	0.4013	t = 2.345, 0.02 > P > .01, S
± SE	0.0916	0.0906	0.079	0.116	
PVA	81.8%	82.3%	89.5%	64.3%	
ϕ	0.606	0.553	0.5368	0.5408	
± SE	0.192	0.221	0.228	0.166	

Int. = intercept
KB = birthrate (cells/cell/hour) = regression coefficient
CPR = cell production rate. Cells/1000 cells/hour
Tca = apparent cell cycle time (hours)
Td = tumour volume doubling time (hours)
tm = mitotic duration (hours)
PVA = percent variability accounted for by regression line
ϕ = cell loss factor
t = t-test comparisons

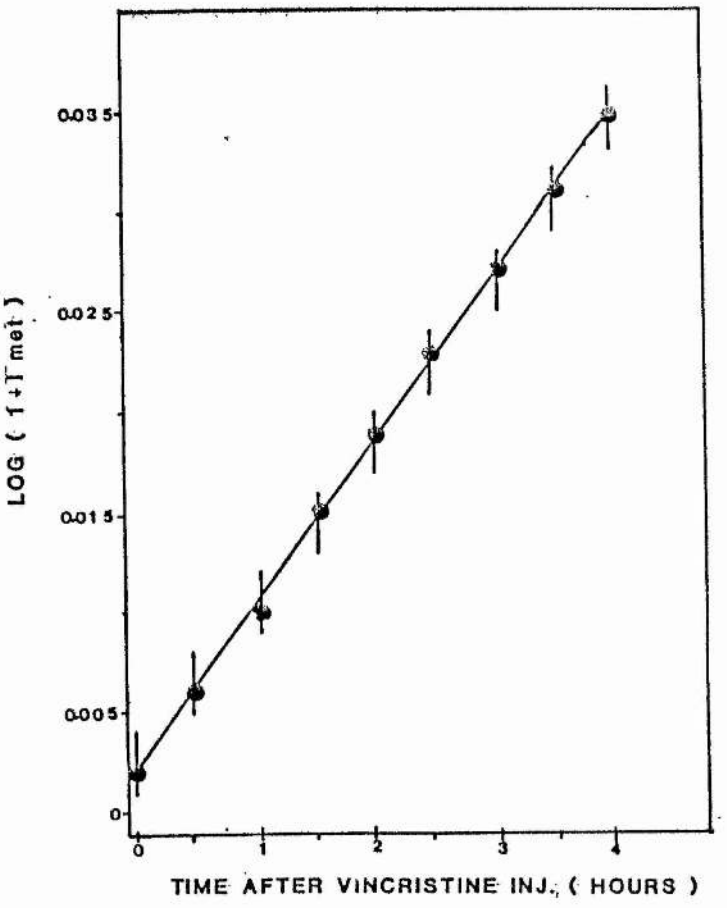


FIGURE 4.2.2.1.5: The metaphase accumulation line for Balb/c plasmacytoma (tumour size 5). The bars indicate 95% confidence limits of the line.

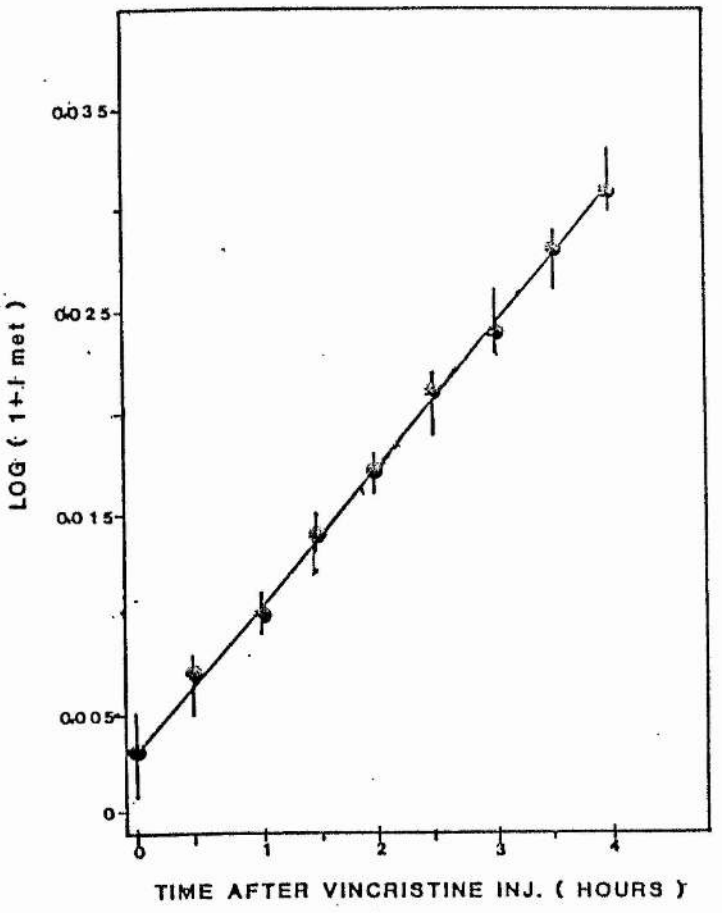


FIGURE 4.2.21.6: The metaphase accumulation line for Balb/c plasmacytoma (tumour size 6). The bars indicate 95% confidence limits of the line.

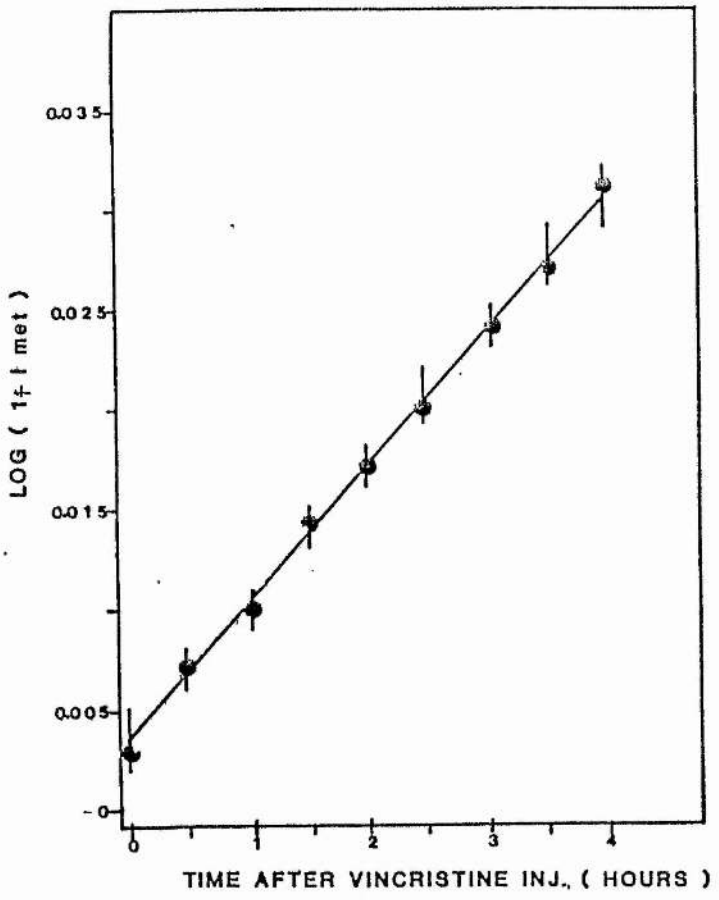


FIGURE 4.2.21.8: The metaphase accumulation line for Balb/c plasmacytoma (tumour size 8). The bars indicate 95% confidence limits of the line.

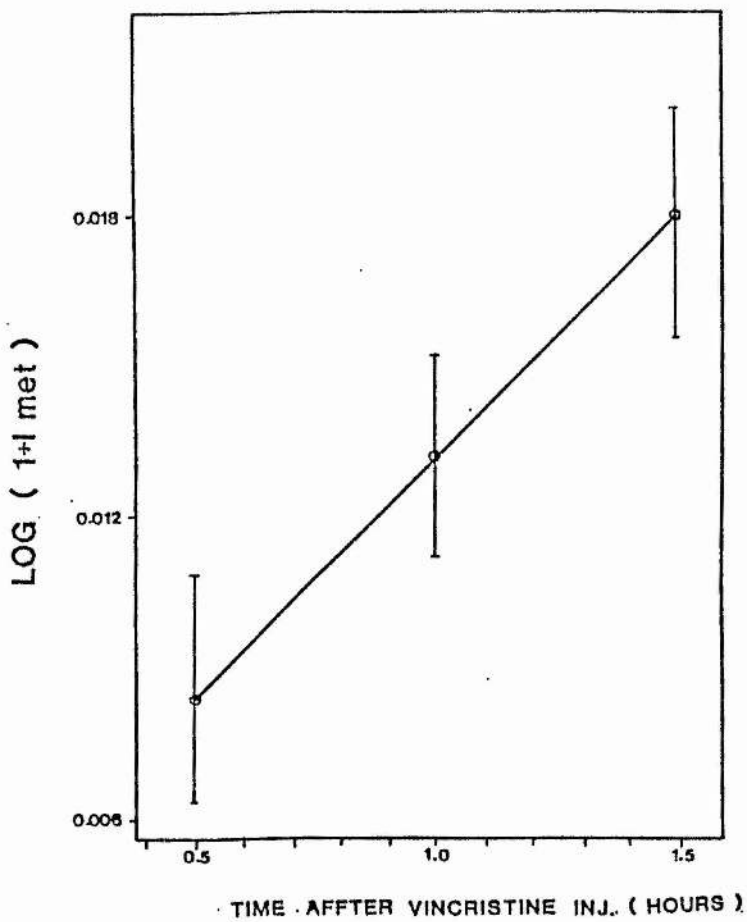


FIGURE 4.2.21.6n: The metaphase accumulation line for Balb/c plasmacytoma (tumour size 6 "nude mice"). The bars indicate 95% confidence limits of the line.

small (1.54 hours) and was insignificant (P>.05).

The cell loss factor ϕ, was nearly constant at the three tumour sizes, with ϕ at 0.55 and 0.53 for tumour size 6 and 8, and slightly higher at size 5 with ϕ at 0.60. The difference in cell loss between the respective tumour sizes (5, 6 and 8) was not significant (P>.05), i.e. (size 5 and size 6 , t = 0.182), (size 5 and size 8, t = 0.223), (size 6 and size 8, t = 0.051).

The metaphase duration (tm) increased with increasing tumour size from approximately 18 minutes at size 5, to 25.5 and 29.7 minutes at tumour sizes 6 and 8 respectively, however, this increase in time was not significant (P>.05) for all comparisons: (tm 5 and tm 6, t = 1.319), (tm 5 and tm 8, t = 1.579), (tm 6 and tm 8, t = 0.583).

The mean cell birth rate at tumour size 6 in "nude" mice was 0.0094 cells/cell/hour which was slightly higher than its counterpart (size 6) in normal Balb/c at 0.00070 cells/cell/hour, which was significant , (0.02>P>0.01). The corresponding mean Tca in "nude" mice was lower than its counterpart in Balb/c (tumour size 6) by 10.5 hours, which was a significant difference (t = 2.908, P<.01). The cell loss in "nude" and Balb/c (tumour size 6) was similar to 0.55 and 0.54 respectively (t = 0.037, P>.05).

The estimates of the respective kinetic parameters (Table 4.2.2.2), were based on the weighted regression analysis. The mean birth rate (KB) for all three tumour sizes, including the "nude" mice (size 6) tumour was relatively less than that described in the unweighted regression analysis, (Table 4.2.2.1). The mean birth rate at tumour size 5 was slightly higher than the mean birth rates in tumour sizes 6 and 8, while the mean birth rate in tumour sizes

TABLE 4.2.2.2 : Metaphase collection function.
B. Weighted regression analysis : Birth rates and related parameters at tumour size 5, 6 and 8 for Balb/c mice and size 6 for nu/nu mice with Balb/c background.

	5	6	8	nu/nu 6	t
Int.	0.003418	0.003352	0.003541	0.004965	KB5 vs KB8
± SE	0.000422	0.000392	0.000220	0.000726	t = 0.187, P > .05 NS
KB	0.007269	0.006633	0.006795	0.008096	KB5 vs KB8
± SE	0.000253	0.000226	0.000148	0.000899	t = 1.617, P > .05 NS
CPR	7.269	6.633	6.795	8.096	KB6 vs KB8
Tca	41.40	45.383	44.304	37.178	t = 0.599, P > .05 NS
± SE	1.442	1.5454	0.967	4.1304	KB6 vs KB6 Nude
tm	0.4701	0.5052	0.5211	0.6132	t = 1.578, P > .05 NS
± SE	0.06	0.061	0.034	0.112	
PVA	84.4%	85.0%	95.0%	60.2%	
ϕ	0.5644	0.5225	0.5333	0.4058	
± SE	0.215	0.235	0.230	0.197	

Int. = intercept
KB = birthrate (cells/cell/hour) = regression coefficient
CPR = cell production rate. Cells/1000 cells/hour
Tca = apparent cell cycle time (hours)
Td = tumour volume doubling time (hours)
tm = mitotic duration (hours)
PVA = percent variability accounted for by regression line
ϕ = cell loss factor
t = t-test comparisons

6 and 8 was similar. The differences between all the respective mean birth rates in tumour sizes 5,6 and 8 were not significant ($P>0.05$). Similarly the difference between the mean birth rate between tumour size 6, growing in athymic mice, and its counterpart tumour size 6 in normal Balb/c mice was not significant ($P>0.05$), although the mean birth rate in the tumour of "nude" mice was slightly higher.

For the "nude" mice, the mean birth rate of their tumours was measured on 3 intervals of time (0.5, 1 and 1.5 hours) as only 6 mice survived the experiment. Therefore, the birth rates described for the tumours growing in athymic "nude" mice, (Tables 4.2.2.1 and 2.2) may probably not be a true estimate, like their counterparts in Balb/c mice where the mean birth rate was estimated from 9 intervals of time, (i.e. from 0.05 hours up to 4 hours in half hour intervals) with a total of 18 mice surviving the experiment. The mean apparent cell cycle time (Tca) for tumour size 6, increased by approximately 4 hours relative to tumour size 5, which was not a significant difference, ($t = 1.887$, $P>0.05$). The mean Tca for tumour size 8 increased by approximately 3 hours relative to tumour size 5, which was not a significant differnce ($t = 1.671$, $P>.05$).

The Tca for tumour size 8 was about 1 hour less than that for tumour size 6, which was not a significant difference ($t = 0.594$, $P>.05$). The Tca for tumour size 6 in "nude" mice was lower than its counterpart tumour size 6 in Balb/c mice by 8.21 hours, however, this difference was not significant ($t = 1.862$, $P>0.05$) due to the large standard error associated with the Tca of the "nude" mice tumour. The metaphase durations (tm) in the respective tumour sizes 5, 6 and 8 growing in normal Balb/c mice and tumour

size 6 growing in athymic mice, were similar at around 30 minutes. The respective t-test comparisons between tm in the respective tumuor groups, [sizes: (5 and 6), (5 and 8), (6 and 8) and (tumour size 6 of Balb/c normals versus 6 "nude") , (t = 0.412, 0.740, 0.230 and 0.845 respectively, P>.05)], were not significant.

Similarly, the cell loss was nearly the same for all tumour groups. The respective cell loss (t-test) comparisons between tumour sizes 5,6 and 8 were not significant, [i.e. (size 5 and size 6, t = 0.132), (sizes 5 and 8, t = 0.1) and (sizes 6 and 8, t = 0.033)]. Similarly the comparison between tumour size 6 growing in "nude" mice and its counterpart tumour size 6 in normal Balb/c mice was not significant (t = 0.184, P>0.05).

In the weighted regerssion analysis the precent variability accounted for by the regression line (PVA) has slightly increased for Balb/c tumours 5, 6 and 8 (in all tumour zones), except for the "nude" mice where the PVA has decreased. The birth rates and their standard errors in the weighted regression analysis for tumour sizes 5, 6 and 8 have decreased, relative to the unweighted regression. The Tca, has increased in the weighted regression, for tumours sizes 5 and 6, but not so, for size 8. The standard error associated with the Tca in the weighted regression was similar to its counterpart [unweighted regression] for tumour sizes 5 and 6, with a relative decrease in size 8, [weighted versus the unweighted regression]. In the "nude" mice, the mean birth rate, in the weighted regression decreased, together with its standard error, relative to the unweighted regression. The Tca has increased in the weighted regression as compared to the unweighted regression, while the standard error has increased in the weighted regression relative to the unweighted regression, (Tables 4.2.2.1

and 2.2).

The results (Tables4.3.1, 2 and 3) for the mean birth rates and the respective Tca and their associated standard error were as follows:

The mean birth rates and their standard errors [weighted regression] in all 3 zones (outer, middle, and inner), of tumour size 5, have decreased as compared to the unweighted regression. The mean Tca has increased accordingly in the unweighted regression together with a small increase in their standard errors, as compared to the weighted regression. Similarly, the respective mean birth rates in all zones of tumour size 6, and their standard errors [weighted regression] have decreased as compared to the unweighted regression, (Table 4.3.2). The respective Tca has increased, [weighted regression] relative to the unweighted regression, although the standard error were similar in both regression models.

The mean birth rates of tumour size 8 in the outer and middle zones and their associated standard errors in the weighted regression have decreased, relative to the unweighted regression, (Table 4.3.3). In the inner zone there was a slight increase in the mean birth rate in the weighted relative the unweighted regression. The mean Tca of the outer zone (weighted) was similar to its (unweighted) counterpart, while the Tca of the middle and outer zones (weighted) were approximately 1 hour different than their (unweighted) counterparts. The standard errors of the respective Tca in the weighted regression were all lower than their counterparts in the unweighted regression.

TABLE 4.3.1 : Metaphase collection function.
1. Unweighted and 2. weighted regression analysis : Birth rates and related parameters for tumour size 5 (outer, middle and inner) zones. Balb/c mice.

1.

	Outer	Middle	Inner	t
Int.	0.002668	0.00302	0.00168	
± SE	0.000851	0.00124	0.00157	KB : outer vs. middle
KB	0.007284	0.008117	0.008714	t = 1.325, P > 0.5 NS
± SE	0.000355	0.000516	0.000654	KB : outer vs inner
CPR	7.284	8.117	8.714	t = 1.921, P > .05 NS
Tca	41.330	37.087	34.546	KB : middle vs inner
± SE	2.0122	2.3571	2.5919	t = 0.721, P > .05 NS
PVA	89.4%	83.1%	77.9%	

2.

	Outer	Middle	Inner	t
Int.	0.003123	0.004041	0.003090	
± SE	0.000591	0.000719	0.000858	KB : outer vs middle
KB	0.006984	0.007248	0.008576	t = 0.466, P > .05 NS
± SE	0.000355	0.000431	0.000515	KB : outer vs inner
CPR	6.984	7.248	7.576	t = 0.947, P > .05 NS
Tca	43.102	41.535	39.734	KB : middle vs inner
± SE	2.190	2.4711	2.6992	t = 0.480, P > .05 NS
PVA	88.5%	84.9%	81.2%	

Int. = intercept
KB = birthrate (cell½/cell/hour) = regression coefficient
CPR = cell production rate. Cells/1000 cells/hour
Tca = apparent cell cycle time (hours)
PVA = percent variability accounted for by regression line
t = t-test comparison

TABLE 4.3.2 : Metaphase collection function.
1. Unweighted and 2. weighted regression analysis : Birth rates and related parameters for tumour size 6 (outer, middle and inner) zones. Balb/c mice.

1.

	Outer	Middle	Inner	t
Int.	0.00297	0.003074	0.00301	
± SE	0.00108	0.000997	0.00110	KB Out. vs KB Mid.
KB	0.006363	0.007341	0.007548	t = 1.586, P > .05 NS
± SE	0.000453	0.000418	0.00046	KB Out. vs KB Inn.
CPR	6.363	7.341	7.548	t = 1.835, P > .05 NS
Tca	47.307	41.008	39.881	KB Mid. vs KB Inn.
± SE	3.3664	2.3363	2.4326	t = 0.333, P > .05 NS
PVA	79.7%	86.0%	84.3%	

2.

	Outer	Middle	Inner	t
Int.	0.002914	0.003536	0.003606	
± SE	0.00074	0.000597	0.000653	KB Out. vs KB Mid.
KB	0.006281	0.006703	0.006915	t = 0.770, P > .05 NS
± SE	0.000426	0.000344	0.000376	KB Out. vs KB Inn.
CPR	6.281	6.703	6.915	t = 1.116, P > .05 NS
Tca	47.926	44.912	43.530	KB Mid. vs KB Inn.
± SE	3.2497	2.3037	2.3650	t = 0.416, P > .05 NS
PVA	81.2%	88.3%	87.1%	

Int. = intercept
KB = birthrate (cells/cell/hour) = regression coefficient
CPR = cell production rate. Cells/1000 cells/hour
Tca = apparent cell cycle time (hours)
PVA = percent variability accounted for by regression line

TABLE 4.3.3: Metaphase collection function.
1. Unweighted and 2. weighted regression analysis Birth rates and related parameters for tumour size 8 (outer, middle and inner) zones. Balb/c mice.

1.

	Outer	Middle	Inner	t
Int.	0.003447	0.003206	0.00352	
± SE	0.000638	0.000904	0.00112	KB Out. vs KB Mid.
KB	0.006613	0.007380	0.006515	t = 0.285, P > .05 NS
± SE	0.000266	0.000377	0.000468	KB Out. vs KB Inn.
CPR	6.613	7.380	6.515	t = 0.036, P > .05 NS
Tca	45.523	40.788	46.206	KB Mid. vs KB Inn.
± SE	1.8316	2.0832	3.3209	t = 1.439, P > .05 NS
PVA	94.5%	91.4%	84.3%	

2.

	Outer	Middle	Inner	t
Int.	0.003255	0.004062	0.003306	
± SE	0.000254	0.000355	0.000455	KB Out. vs KB Mid.
KB	0.006572	0.007114	0.006697	t = 1.844, P > .05 NS
± SE	0.000171	0.000239	0.000307	KB Out. vs KB Inn.
CPR	6.572	7.114	6.697	t = 0.355, P > .05 NS
Tca	45.803	42.313	44.948	KB Mid. vs KB Inn.
± SE	1.1919	1.4237	2.0614	t = 1.071, P > .05 NS
PVA	97.6%	96.1%	92.9%	

Int. = intercept
KB = birthrate (cells/cell/hour) = regression coefficient
CPR = cell production rate. Cells/1000 cells/hour
Tca = apparent cell cycle time (hours)
PVA = percent variability accounted for by regression line

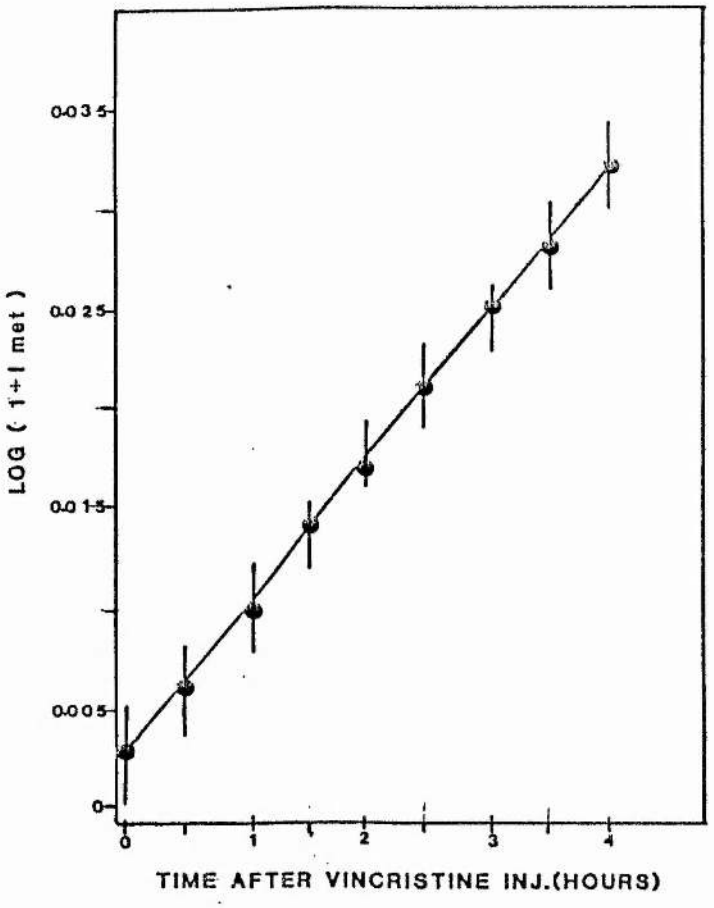


FIGURE 4.3.1.1: The metaphase accumulation line for the outer zone of Balb/c plasmacytoma (tumour size 5). The bars indicate 95% confidence limits of the line.

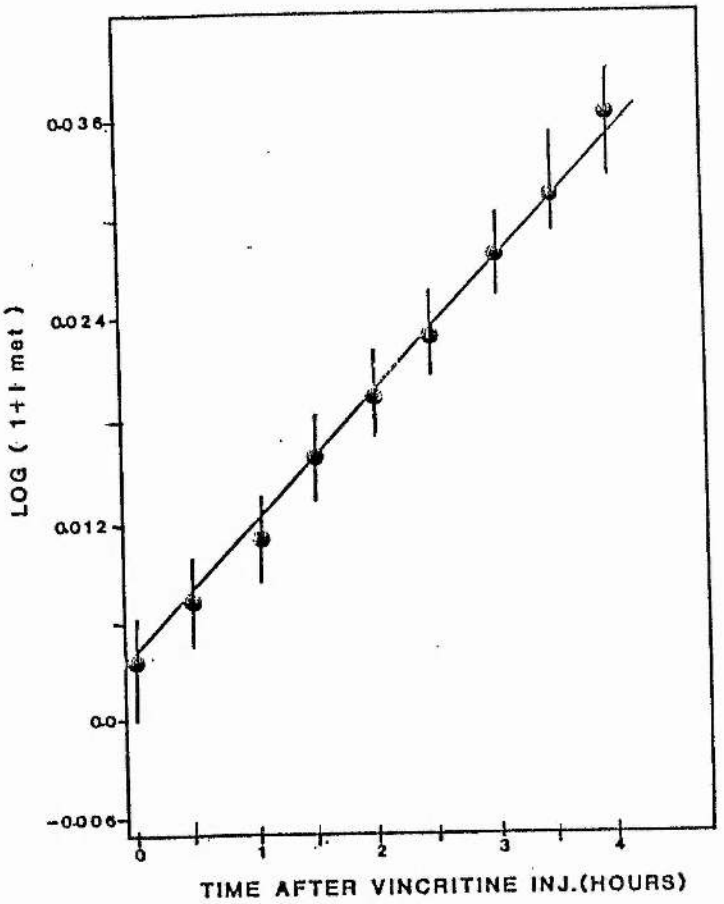


FIGURE 4.3.1.1: The metaphase accumulation line for the middle zone of Balb/c plasmacytoma (tumour size 5). The bars indicate 95% confidence limits of the line.

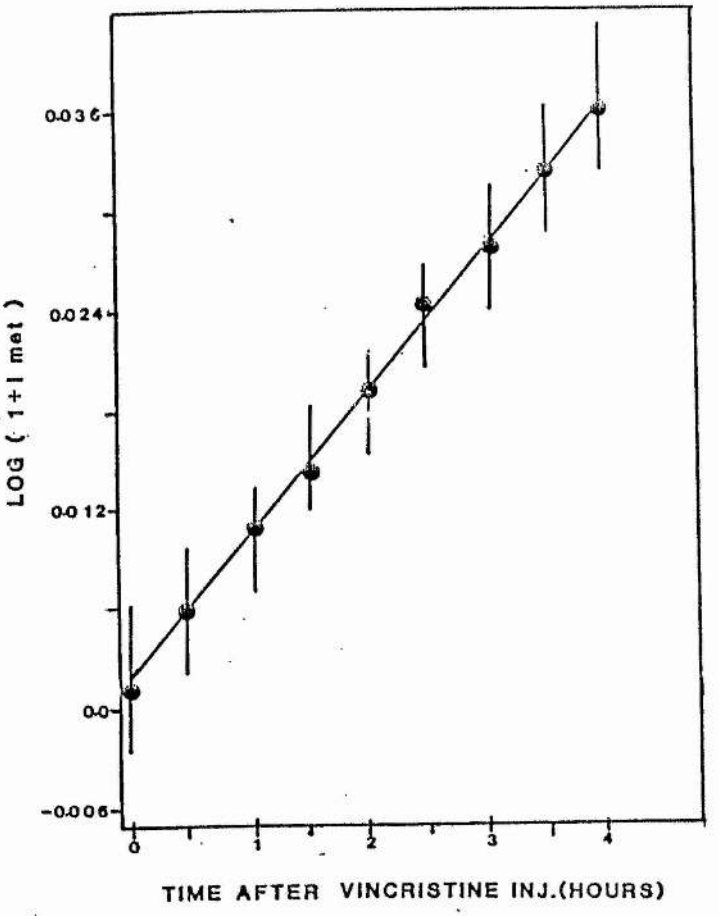


FIGURE 4.3.1.1: The metaphase accumulation line for the inner zone of Balb/c plasmacytoma (tumour size 5). The bars indicate 95% confidence limits of the line.

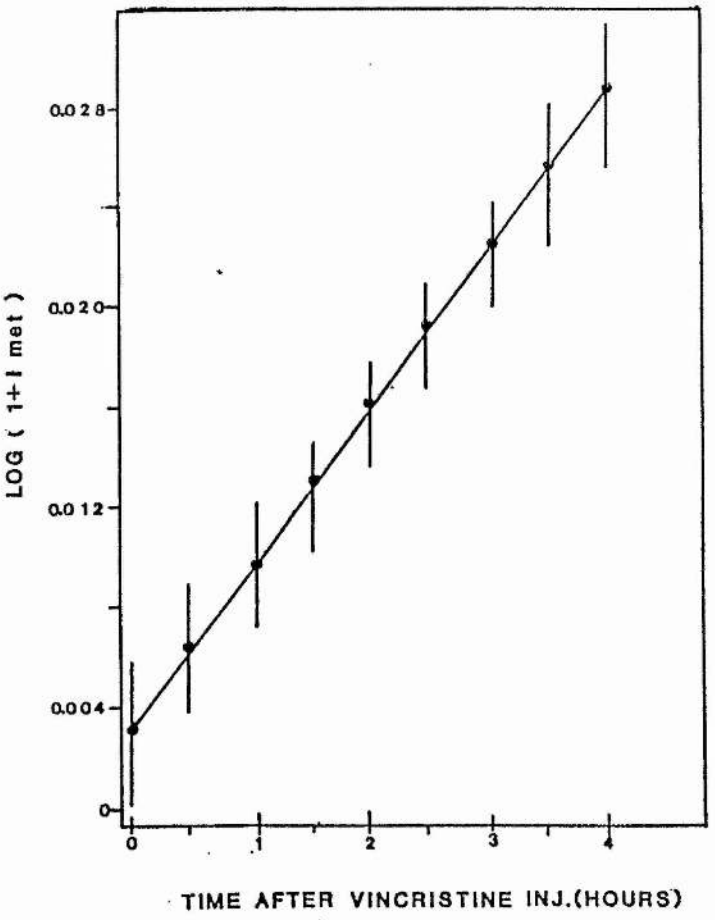


FIGURE 4.3.2.1 : The metaphase accumulation line for the outer zone of plasmacytoma (tumour size 6). The bars indicate 95% confidence limits of the line.

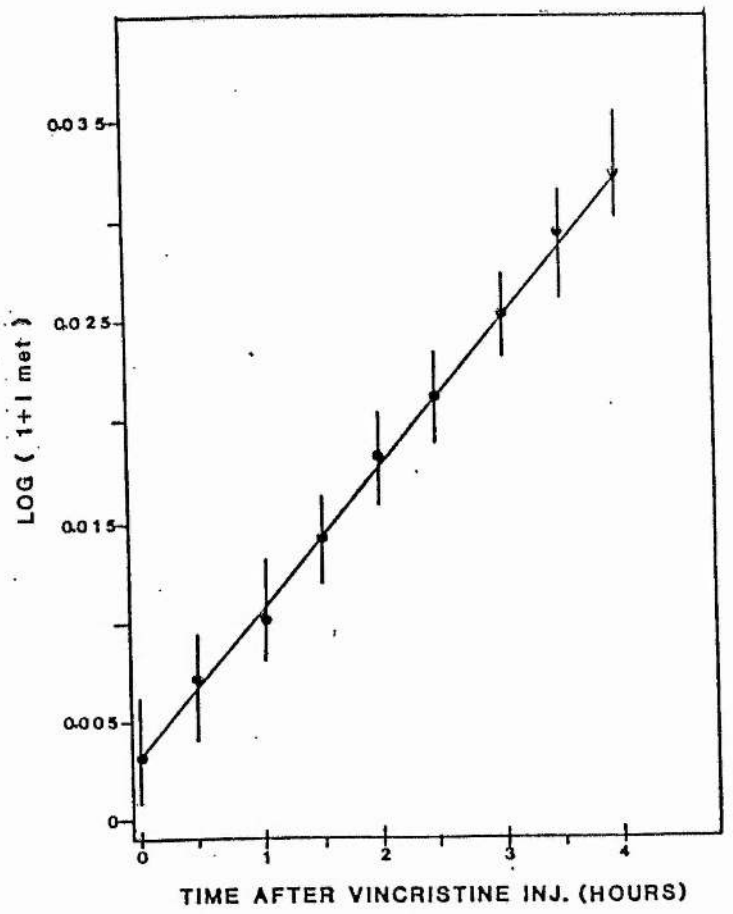


FIGURE 4.3.2.1: The metaphase accumulation line for the middle zone of plasmacytoma (tumour size 6). The bars indicate 95% confidence limits of the line

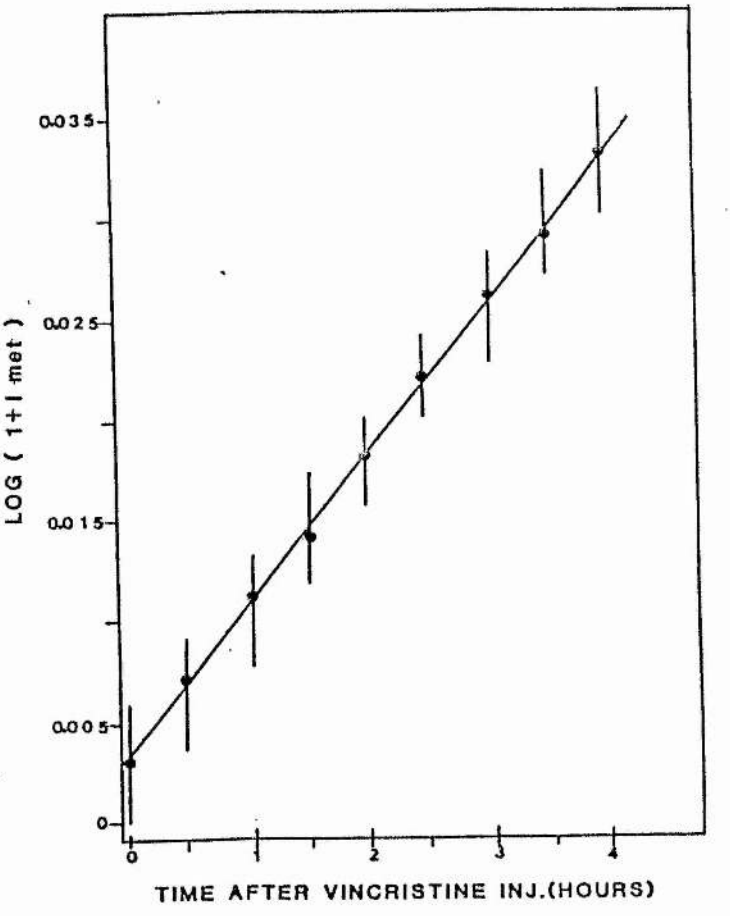


FIGURE4.3.2.1: The metaphase accumulation line for the inner zone of plasmacytoma (tumour size 6). The bars indicate 95% confidence limits of the line.

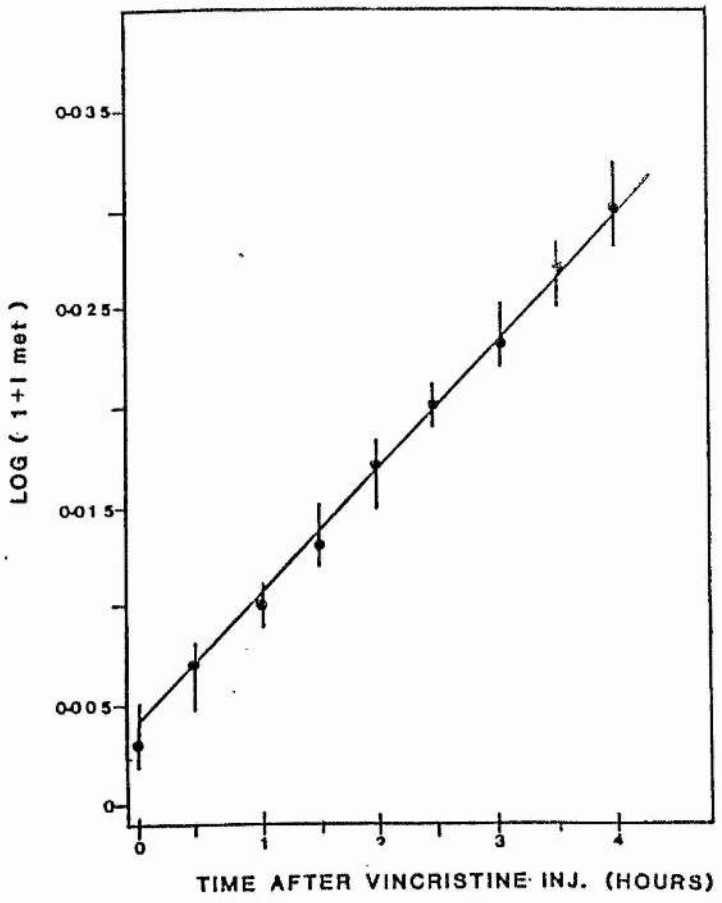


FIGURE 4.3.3.1: The metaphase accumulation line for the outer zone of plasmacytoma (tumour size 8) . The bars indicate 95% confidence limits of the line.

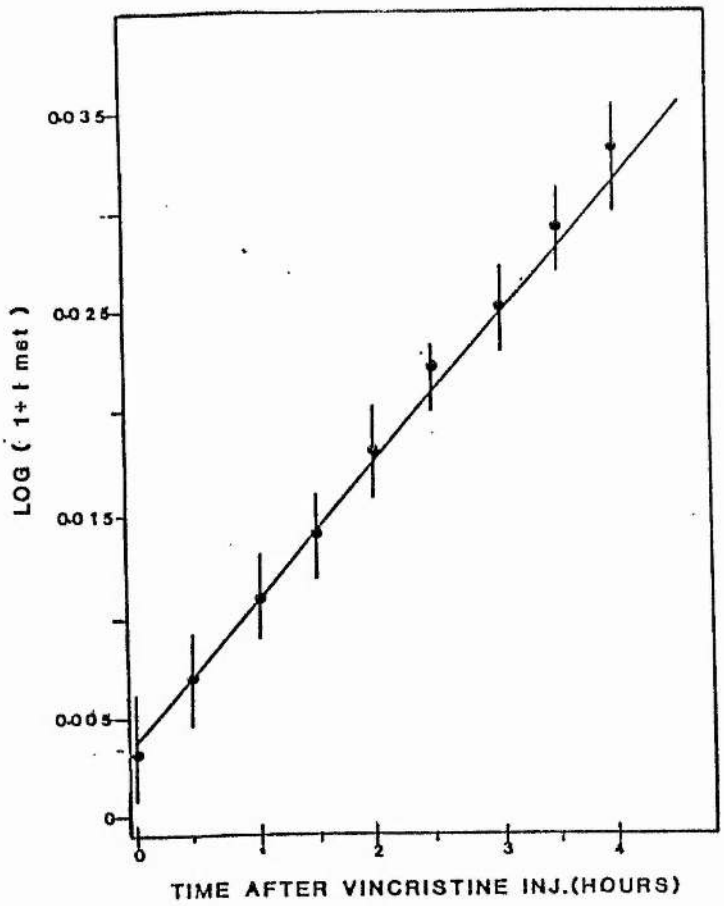


FIGURE 4.3.3.1: The metaphase accumulation line for the middle zone of plasmacytoma (tumour size 8). The bars indicate 95% confidence limits of the line.

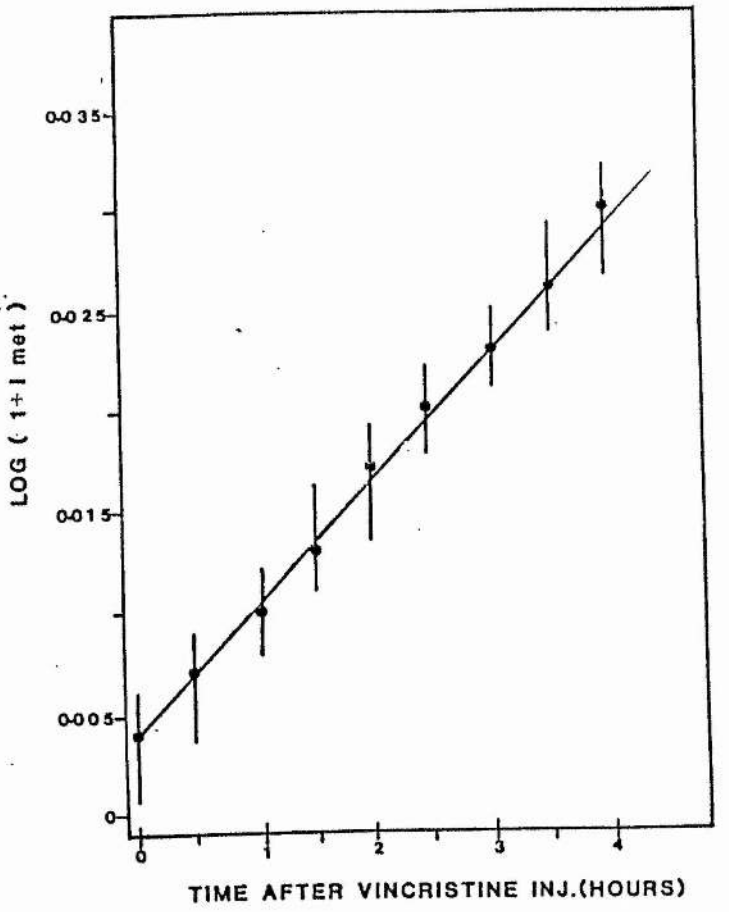


FIGURE 4.3.3.1: The metaphase accumulation line for the inner zone of plasmacytoma (tumour size 8). The bars indicate 95% confidence limits of the line.

The mean birth rates in all three zones of tumour size 6 growing in "nude" mice and their associate standard errors, in the weighted regression were lower than their unweighted counterparts (Table 4.3.4). Accordingly, the respective mean Tca in the weighted were higher than their unweighted counterparts. However, the standard error for the respective Tca in the weighted regression were all relatively higher than their unweighted counterparts.

The mean birth rates in the inner and middle zones [unweighted] were slightly higher than the outer zone (Table 4.3.1), although the difference between the three zones was not significant ($P>.05$). Similarly the mean Tca decreased from the periphery to the centre of tumour by approximately 7 hours, although the respective differences in Tca for each of the two tumour zones was not significant ($P.>05$) except for one comparison, i.e. tumour size 5 (outer and middle zones, $t = 1.375$), (outer and inner zones, $t = 2.072$, $0.05>P>.02$), (middle and inner zones, $t = 0.727$). In the weighted regression, again, there was a slight increase in the mean birth rate from the periphery to the centre of the tumour which was not significant ($P>.05$), together with an associated decrease in Tca from the periphery to the centre by approximately 3 hours, which was also not significant ($P>.05$),i.e (outer and middle zones, $t = 0.475$), (outer and inner zones, $t = 0.973$) and middle and inner zones, $t = 0.493$).

The mean birth rates in the unweighted regression in the inner and middle zones of tumour size 6 were slightly higher than the outer zone, (Table 4.3.2), although the differences between the respective zones were not significant. Similarly, the mean Tca decreased from the periphery to the centre of the tumour by 7

TABLE 4.3.4 : Metaphase collection function.
1. Unweighted and 2. weighted regression analysis : Birth rates and related parameters for tumour size 6 (outer, middle and inner) zones. nude mice with Balb/c background.

1.

	Outer	Middle	Inner	t
Int.	0.003158	0.00366	0.00453	KB:Out. vs Mid.
± SE	0.000866	0.00194	0.00214	t = 0.134, P > .05 NS
KB	0.008679	0.00995	0.00963	KB: Out. vs Inn.
± SE	0.000802	0.00180	0.00198	t = 0.445, P > .05 NS
CPR	8.679	9.95	9.63	KB: Mid. vs Inn.
Tca	34.681	30.251	31.256	t = 0.119, P > .05 NS
± SE	3.204	5.472	6.426	
PVA	87.2%	63.5%	57.0%	

2.

	Outer	Middle	Inner	t
Int.	0.003763	0.00519	0.00594	KB : Out. vs Mid.
± SE	0.000631	0.00139	0.00138	t = 0.125, P > .05 NS
KB	0.008002	0.00824	0.00805	KB : Out. vs Inn.
± SE	0.000783	0.00172	0.00171	t = 0.025, P > .05 NS
CPR	8.002	8.24	8.05	KB ; Midd. vs Inn.
Tca	37.620	36.550	37.392	t = 0.078, P > .05 NS
± SE	3.679	7.636	7.9314	
PVA	85.9%	56.3%	55.5%	

Int. = intercept
KB = birthrate (cells/cell/hour) = regression coefficient
CPR = cell production rate. Cells/1000 cells/hour
Tca = apparent cell cycle time (hours)
PVA = percent variability accounted for by regression line

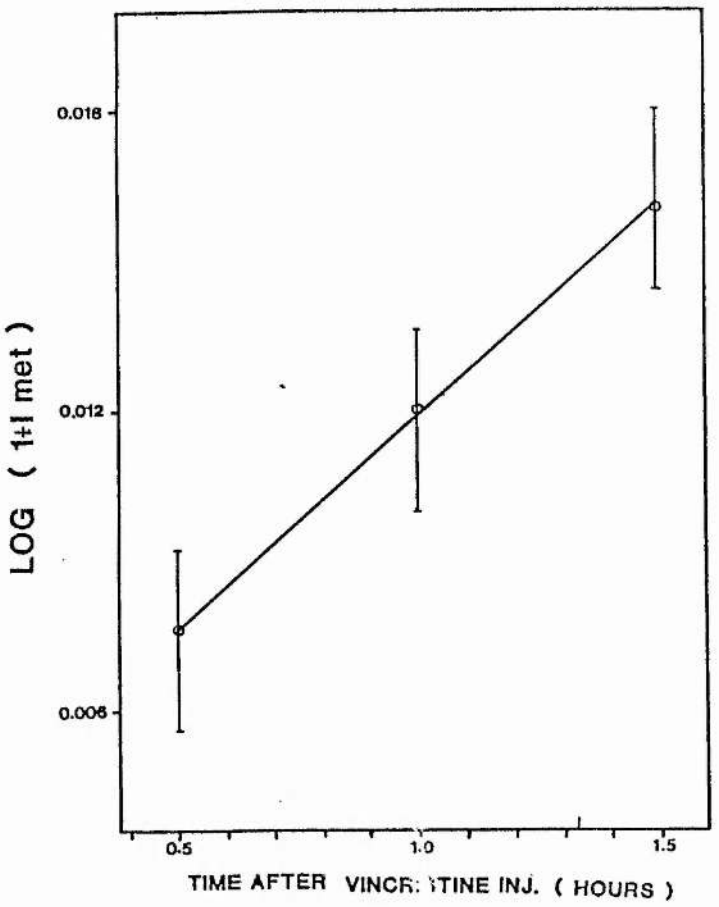


FIGURE 4.3.4.1 : The metaphase accumulation line for the outer zone of plasmacytoma (tumour size 6 "nude mice"). The bars indicate 95% confidence limits of the line.

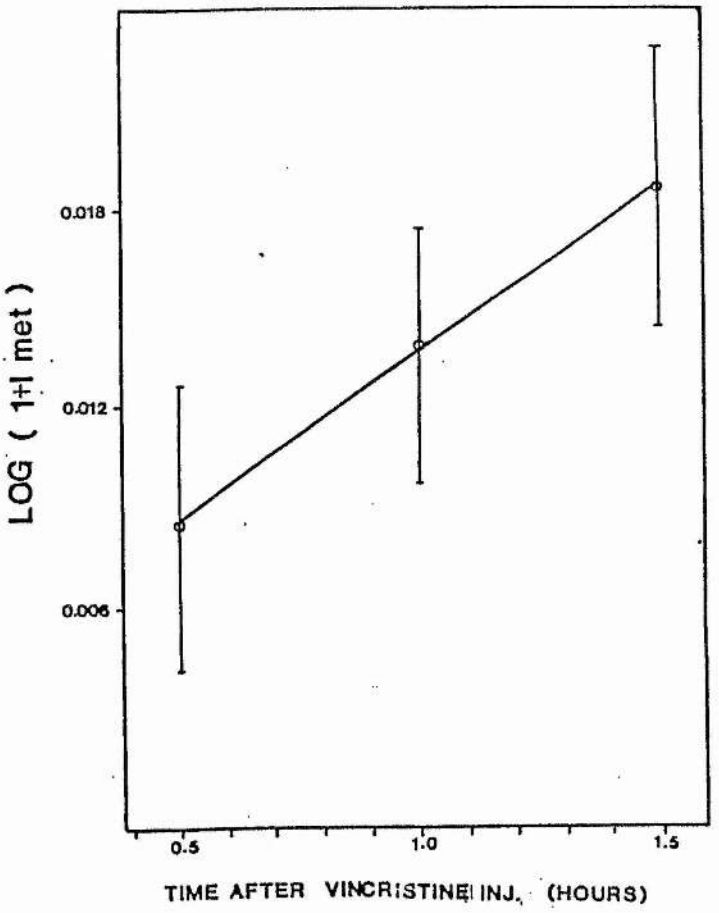


FIGURE 4.3.4.1 : The metaphase accumulation line for the middle zone of plasmacytoma (tumour size 6 "nude mice"). The bars indicate 95% confidence limits of the line.

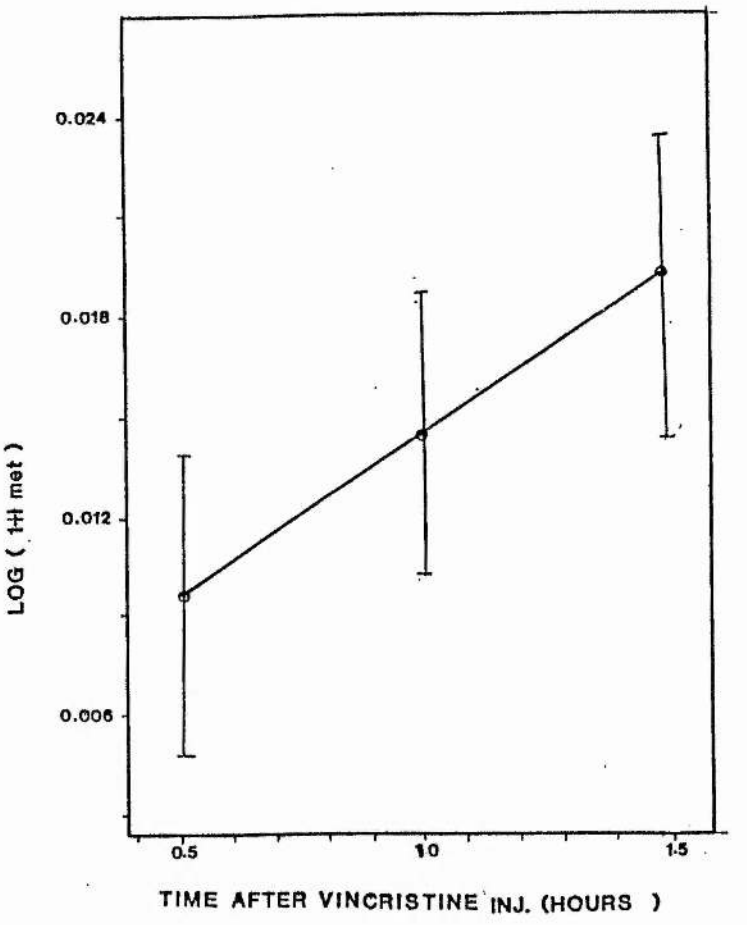


FIGURE 4.3.4.1 : The metaphase accumulation line for the inner zone of plasmacytoma (tumour size 6 "nude mice"). The bars indicate 95% confidence limits of the line.

hours, although the differences between the respective mean Tca for each zone were not significant (P>.05), i.e. (outer and middle zones. t = 1.540), (outer and inner zones, t = 1.792) and (middle and inner zones, t = 0.758).

In the weighted regression the mean birth rate was nearly similar in the 3 zones (P>.05). The Tca is sensitive to a change in the regression coefficient (KB) and the slightest change in KB can lead to a magnified difference in Tca. Thus the difference between (KB : outer and inner zones) is 0.000634 units which has accounted for about 4 hours difference in Tca between these 2 zones. The mean Tca has decreased from the periphery to the tumour centre, but the respective differences between each zone were not significant (P>.05). (outer and middle zones, t = 0.758), (outer and inner zones, t = 1.095), (outer and inner zones, t = 0.419).

The mean birth rate [unweighted regression] in the zones of tumour size 8 fluctuated slightly, (Table 4.3.3) with the outer and inner zones being similar in KB and both were slightly less than the middle zone, although these differences were not significant (P>.05). Thus the mean Tca in the inner zone was approximately one hour more than the outer zone, and the middle zone was approximately 5 hours different than either adjacent zone. However, these differences were not significant (P>0.05), (outer and inner zones, t = 1.717), (outer and inner zones, t = 0.179), (middle and inner zones, t = 1.384). Similarly, the same was true in the weighted regression for KB as in the unweighted regression, where the differences between the respective zones were not significant, (P>.05). Also, the difference between the respective mean Tca for each zone was not significant (P>.05),(outer and middle, t = 1.887), (outer and inner zones, t = 0.362) and (middle

an inner zones, t = 1.04).

The mean birth rates in the middle and inner zones [unweighted regression] of tumour size 6 growing in "nude" mice were relatively higher than the outer zone, whereas in the weighted regression they were almost similar, (Table 4.3.4). The differences in the mean birth rates between the respective zones [unweighted and weighted regressions] were not significant (P>.05).

The mean Tca in the unweighted regression outer zone was relatively higher than either the middle or inner zones by approximately 4 to 3 hours respectively; however, the difference in Tca between the respective zones was not significant (P>.05), (outer and middle zones, t = 0.699), (outer and inner zones, t = 0.478), and (middle and inner zones, t = 0.118).

The mean Tca in the weighted regression was essentially the same for all 3 zones, except for the middle zone which was approximately 1 hour shorter than either adjacent zone. The respective differences in Tca between the respective zones were not significant (P>.05), (outer and middle zones, t = 0.126), (outer and inner zones, t = 0.029) and (middle and inner zones, t = 0.076).

As described above, (section 3.4.2.2) the kinetics of tumour growth were assessed in normal and "nude" mice. Tumour growth was assessed in terms of cell production rate, apparent cell cycle time and cell loss factor at various stages of tumour growth.

The results have demonstrated that the birth rate has decreased as the tumour grew larger (a function of tumour size or age). Consequent with this reduction in the birth rate, the apparent cell cycle time has increased. The cell loss factor was

generally similar at the various stages of tumour growth.

The birth rate in the "nude" mice was relatively higher than its counterpart in normal mice, although the cell loss factor was similar in both groups.

The respective birth rates and apparent cell cycle times were examined in terms of a weighted and unweighted regression analyses, to assess the contribution of the weighted analysis (as compared to the unweighted analysis), to the reduction of the standard error associated with the respective apparent cell cycle time. The results have demonstrated that the use of the weighted analysis variant was useful in reducing the standard errors in some curves, and was not a general effect.

The respective birth rates were also assessed in terms of tumour zones, to know whether the birth rate varied between the tumour's periphery and centre. Tumour centres are usually more necrotic than peripheral areas which have a relatively better supply of blood than central areas. The results have demonstrated that the birth rate did not decrease as a function of tumour zone, from the peripheral areas to the central areas. In the plasmacytoma tumour, reported in this work, the distribution of necrosis was similar among the respective tumour zones.

TABLE 4.3.5: The percentage of cells in normal Balb/c mice tumours size 5, 6 and 8 in their respective zones (outer, middle and inner).

	% cells per zone			
Tumour Size	Outer	Middle	Inner	Tumour Mean
5	72.87	76.78	76.20	75.32
6	77.13	76.50	75.72	76.45
8	76.78	77.59	75.26	76.54
Zone Mean	75.62	76.96	75.72	

Analysis of variance of Table 4.3.5

Stratum	DF	SS	MS	F	P
Environment	2	150.3	75.1	0.246	> .05, NS
Zone	2	178.6	89.3	0.293	> .05, NS
Envir. Zone	4	483.0	120.7	0.396	> .05, NS
Residual	477	145557.7	305.2		
TOTAL	485	146369.5			

NS = not significant
S = significant

CHAPTER FOUR

DISCUSSION

In vitro systems are often used to assess the effects of regulators of cellular proliferation. It is necessary to evaluate the culture conditions when using these types of assays as many factors can affect cellular proliferation. The effects of culture environment have been investigated using a murine plasmacytoma (MPC-11). Most cultured cells show an absolute dependence on serum for growth (Holley 1974) (Lanks and Kasambalides 1980), as apart from the basal nutrient mixture of salts, sugars, aminoacids and vitamins, which are required by the cultures, serum is essential for cell survival as it contains hormones, nutritional factors and stromal elements, and proteins, (Barnes and Sato 1980). Essentially, the serum can provide a complex of hormones that are growth stimulatory for a given cell type (Mather 1984), as Hyashi and Sato (1976) have shown in the pituitary cell line, which was grown in serum-free medium supplemented with hormones, growth factors and transferrrin. A variety of sera have been used as supplements for in vitro cultures, the most popular of which is FCS (foetal calf serum). However, when FCS is used in conjunction with assays to assess cellular DNA synthesis , using labelled precursors such as 3H-Tdr or 125IUDR, FCS can influence DNA synthesis.

When plasmacytoma cultures were cultured at various cell concentrations per well, there was a good relationship between cell dose and uptake of 125IUDR, (Figures/Tables 1.1.1 and 1.1.2) and there was a higher uptake of isotope by cell cultures cultured without FCS than with active or heat decomplemented FCS (FCS-HI heat inactivated). The inhibition of isotope uptake by active FCS was more than that

produced by FCS-HI (Table 1.6.2), and this may correspond to the inactivation of the complement proteins (Goetze and Muller-Eberhard 1976) which may bind to antigen-antibody complexes, activating the classical pathway, or without the requirement for antibody (except IgA) to activate the alternate pathway and thus perturbing or damaging the cell's membrane (Brown and Frank 1981), (Weiler et al., 1982). Kierszenbaum and Budzko (1977) have described the cytotoxic effects of normal sera (human, rabbit and guinea pig) on lymphoid cells which were antibody independent, as they were affected through the alternate pathway. Thus both C4-deficient guinea-pig serum and C2 deficient human serum displayed cytotoxicity on target cells. Sera from all three types were active in the absence of free Ca^{2+} which was required to activate complement via the classical pathway , although when the sera were heated for 20 minutes at 56 °C to destroy the activity of factor B of the alternate pathway the cytotoxic activity was lost. Eidinger et al., (1977) have shown that human serum induces cytolysis of mouse thymus and thymoma cells and cytostasis of bone marrow and spleen cells. Decomplementation procedures have demonstrated that the heterocytotoxic effects were mediated in part by the alternate pathway of complement. Similarly, the activation of the alternate pathway in FCS by mouse thymocytes were reported by Eisenberg et al., (1979). The activation of the alternate pathway by human lymphoblastoid B and T cell lines has also been demonstrated by Praz and Lesavre (1983). The inhibition of 125IUDR uptake was maintained, even when the cell cultures were pre-incubated for various lengths of time before the addition of 125IUDR , (Figure/Table 1.7). Furthermore, the inhibition of isotope uptake by FCS was dose dependant, when the FCS concentration was increased, the uptake of isotope decreased, (Tables 1.2.1 and 1.2.2.1A, Figure 1.2.2.1).

The uptake of 125IUDR by DNA was assessed in two ways; First, was the estimation of isotope uptake in DNA extracted from plasmacytoma cultures, cultured in several FCS-HI concentrations, and the second, was through the specific inhibition of 125IUDR uptake by hydroxyurea. The amount of isotope incorporated into DNA decreased with increasing serum concentration, and this was corroborated when the acid insoluble and acid soluble fractions were extracted and assessed for isotope uptake as described in Figure/Table 1.5.1.1. The effects of FCS on the amount of isotope incorporated by cell cultures is discussed in the lipoprotein section.

Hydroxyurea is a phase specific cytotoxic substance, specifically inhibiting the uptake of isotope by cell cultures in S phase, (Young and Hodas 1964), (Young et al., 1967), (Krakoff et al., 1968) (Iversen et al., 1982), (Shu and Hoshino 1983) (Dormer and Bohmer 1984). The uptake of 125IUDR was effectively blocked (>90% uptake) by hydroxyurea at doses between 10^{-3} and 10^{-1} moles as described in Figure/Table 1.5.1.2. This was similar to the results of Riches et al., (1976) using CBA mammary adenocarcinoma in a 4-hour culture with hydroxyurea. Gerald and Neumuller (1983) using Hela S3 cells have shown that 3H-Tdr incorporation into DNA was inhibited (90%) by 2.5 x 10^{-3} moles in a 3-hour incubation period. Larson and Yachnin (1983) have shown that 1m.mole of hydroxyurea was required to abolish the 14C-thymidine uptake by chronic leukemic cells cultured with cytochalasin B. Peiffer and Tolmach (1967) using Hela cell cultures, have shown that the uptake of isotope was inhibited by hydroxyurea with a concentration between 2 x 10^{-4} moles and 10^{-3} moles, with similar estimates by Young et al., (1967) of 1 x 10^{-4} moles to 10^{-3} moles, and Young and Hodas (1964) of 10^{-4} moles to 10^{-3} moles. Similarly, Sinclair (1965) has shown that the uptake of 3H-Tdr by Chinese hamster lung cells was blocked by hydroxyurea at a concentration of 5 x 10^{-4} to 10^{-3}

moles after a 1-hour culture period.

Iododeoxyuridine an analogue of thymidine (Pursoff 1959), has been shown to be utilised as an isotope, exclusively into DNA of proliferating cells (Hofer and Hofer 1971, Kelly et al., 1981), and is an adequate and stable tracer for studying DNA metabolism and cellular kinetics (Commerford 1965). The reutilisation of 125IUDR is very low compared to 3H-Tdr where reutilisation is greater after release from dead cells (Hughes et al., 1964, Dethlefsen 1970, 1971, 1974, Hofer and Hofer 1971). 125IUDR has been used extensively for measurements on cell proliferation (Bonnmassar et al., 1975), (Riches et al., 1981) and cell loss in vitro (Dethlefsen 1971, Dethlefsen and Riley 1973, Dethlefsen et al., 1977, Begg 1977, Franko and Kallman 1980, Kelly et al., 1981, Porschen et al., 1983); as a cellular label to monitor cell distribution in various organs (Hofer et al., Joel et al., 1977, Ottaway et al., 1983, Spach and Motta 1983,); as a cellular label to assess DNA synthesis in vivo (Siegers et al., 1979, Rijke et al., 1981) and in vitro (Riches et al., 1976, Bonmassar et al., 1978, Mistry et al., 1983, Buchanan 1984, Nelson et al., 1984), and for cytotoxicity assays (Oldham et al., 1973, le Mevel et al., 1973, Ting et al., 1977, Evans and Eidlen 1983). However, 125IUDR is chemically toxic (Cheong et al., 1960, Morris and Cramer 1966, le Mevel et al., 1973, and radiotoxic (Mak and Till 1963, Hofer and Hughes 1971, Porteus 1971, le Mevel et al., 1973, Burki et al., 1973), thus it is important to use low doses of this isotope in experimental assays. Cheong et al., (1960) have shown that there was an inhibition of growth in in vitro cell cultures of H.E.P. cells at a concentration of 1-5 ug/ml of IUDR (30% inhibition at 5ug/ml). Morris and Cramer (1966) showed that $1\text{-}2\times10^{-4}$ moles IUDR inhibited cell proliferation after one cell division. Cold IUDR at a concentration of 1.41×10^{-5} moles incubated with MCA-10 cells for 18 hours; inhibited the division

of cells up to 91 hours after the incubation with cold IUDR (le Mevel et al., 1973).

le Mevel et al., (1973) using 0.5 uci per 100ul per culture well (stock solution 50 uci/ml containing 0.005 mg/ml IUDR) have shown that the uptake of 125IUDR was linear with time between 6 to 18 hours, and thereafter declines up to 72 hours, and have also shown that as the concentration of isotope increased (5uci per 100 ul per culture well), the 125IUDR toxicity for the cell cultures increased. le Mevel et al., (1973) have shown that the MCA5 and MCA10 cell lines (explants from 3- methylcholanthrene induced sarcomas) appeared to be more damaged by isotope (0.5uci/well) than the MCA-2 cell line and the normal cell lines (normal muscle C57BL/6 and C3H/F mouse cell lines), between 30-48 hours incubation period.

Riches et al., (1976) have used 1 uci of 125IUDR (sp.act 1-6 mci/mg) per 3ml culture medium which was equivalent to a maximum of 0.333ug per ml of IUDR ($\simeq 1 \times 10^{-6}$ moles), for a 24 hour culture period, and was shown to be not cytotoxic for CBA mammary adenocarcinoma explants. In the experiments reported in this thesis, the 125IUDR used (500uci per 0.5ml;sp.act. 5ci/mg.) was at a concentration of 0.2 uci per 200ul culture medium per well which was equivalent to 2×10^{-4}ug/ml or 5.64×10^{-10} moles, which was unlikely to be cytotoxic for a 4 hour or a 16 hour incubation period. Porteus (1971), using 125IUDR of similar specific activity to that of Riches et al., (1976) has found no effect on the growth of BP8 cells cultured for 17 hours with 0.03 uci/ml, but with 0.1 to 1uci/ml, there was some reduction in growth.

Plasmacytoma cultures were also incubated with 3H-Tdr, to assess DNA synthesis in relation to serum effects, (Figure/Table 1.3). There was a similar inhibition of 3H-Tdr uptake by the cell cultures with increasing serum concentration. There are pitfalls in 3H-Tdr, used as

an isotope to measure cell proliferation (Laurence et al., 1979), and these have been reviewed by Maurer (1981). 3H-Tdr affected clonal growth of Hela S3 at a concentration of 0.1uci/ml (sp.act;1.88ci/mmole), (Drew and Painter 1959), while Weizsacker et al., (1981) using a similar specific activity reported an inhibition of 9L rat gliosarcoma cell growth at a concentration of 0.2uci--10uci/ml 3HTdr. 3H-Tdr also affected the progression of cells through the cell cycle (Ehmann et al., 1975, Marz et al., 1976, Darzynkiewicz et al., 1984). However, 3H-Tdr is more efficiently utilised by Thymidine kinase, [ATP; thymidine-5'-phosphotransferase E.C.2.7.1.2.1], (Cleaver 1967, Baugnet-Mahieu and Goutier 1968, Webb et al., 1980), this is because more thymidine di-phosphates and tri-phosphates are formed with thymidine (3H-Tdr) than with Iododeoxyuridine (125IUDR), (Baugnet-Mahieu and Goutier 1968). The fact that IUDR is much less phosphorylated to the tri-phosphate stage than thymidine, decreases the probability of its reutilisation, and justifies the use of 125IUDR as a suitable marker, (Baugnet-Mahieu and Goutier 1968).

The inhibitory effects of FCS on isotope uptake by cell cultures were selective, as FCS interacted differently with each cell type, in terms of FCS concentration and the period of incubation. Thus for plasmacytoma cultures (Tables 1.2.2.1A and 1B) at all concentrations of FCS, there was inhibition of isotope uptake, although the relative amount of isotope uptake inhibition was decreasing at later periods of incubation.

For the mammary adenocarcinoma cultures as described in Figure/Table 1.2.2.2 there was a stimulation of isotope uptake rather than inhibition, at some serum concentrations and incubation periods, although the relative amount of isotope uptake inhibition was

increasing at later periods of incubation.

For the normal thymocytes, as described in Figure/Table 1.2.2.3, the effects of FCS were opposite, that is, at 5-10% FCS there was a stimulation of isotope uptake at any incubation period, while at 15-25% FCS there was an inhibition of isotope uptake, which was fairly constant at 15%, while at 25% the inhibitory effects decreased as the incubation period increased. One common feature among all these cultures was an increasing inhibitory effect on isotope uptake as the FCS concentration increased in each incubation period.

However, FCS may also be inhibitory to normal thymocytes at a concentration of 10%, in fact, more inhibition of isotope uptake was produced on thymocytes than on plasmacytoma cultures with a certain batch of FCS as described in Tables 1.6.1.1 and 1.6.1.3. The effects of FCS on the uptake of isotope by cell cultures were variable, as different batches of FCS produced different inhibitory effects as described in Table 1.4. Honn et al., (1975) have shown that different batches of FCS were variable as to their contents of ions, cholesterol, hormones, proteins and albumin. In some batches, the variability was quite large. Bovine serum albumin and human serum albumin were equally inhibitory on isotope uptake by plasmacytoma cultures, as described in Tables 1.8.2.A and B. Rat, mouse, horse and human sera were equally inhibitory on isotope uptake even when they were heat inactivated, as described in Tables 1.8.1 and 1.8.2.B.

The inhibitory effects of FCS on isotope uptake by cell cultures were not due to a non-specific effect of isotope activity remaining in the wells as a function of serum concentration as described in Table 1.9; i.e.: as the serum concentration in the cell cultures increased, the amount of isotope remaining in the cell culture wells decreased.

The interaction of BSA with plasmacytoma cultures at two different cell culture densities was similar, that is, (there was not an increase in isotope uptake inhibition at high compared to low cell densities, (Table 1.6.1.2). FCS interacted differently with normal thymocytes (Table 1.6.1.3) producing a small increase in isotope uptake inhibition at a higher cell density, and in the plasmacytoma cultures (Table 1.6.1.1), the inhibition was higher at a higher cell density, although these inhibitory effects in the case of plasmacytoma varied with the FCS batch and incubation period, but as described in Table 1.6.2 there was a marked inhibition of isotope uptake at a high cell density in the presence of FCS, than without FCS, in an extended incubation period. The plasmacytoma were probably able to sustain their proliferation for an extended period of incubation without the requirement for serum, as tumour cells have been known to produce their own peptide growth factors, (Sporn and Roberts 1985). The labelling index of plasmacytoma cells cultured in FCS as described in Table 2.5 was slightly reduced, although not significantly as compared to cultures without FCS. Thus FCS had no significant inhibitory effect on cells in S-phase, or on the passage of cells from G_1 to S. However, some constituents of serum such as lipoproteins, may block the flow of cells from G_1 to S-phase, but this was not evident in the incubation sequence described in Figure/Table 1.7, although there was a decrease in uptake of isotope by cell cultures in some incubation sequences with different batches of FCS as described in Figure/Table 2.7 and Figures/Tables 3.1.3A and 3B. This may be due to the effects of lipoproteins or the interaction of polyamine oxidase with polyamines as will be described below.

FCS contains polyamine oxidase, an enzyme which converts spermine and spermidine into their respective aldehydes, which can interfere with DNA synthesis, (Bachrach and Reches 1966, Higgins et al., 1969, Allen et al., 1979, Gaugas 1980, Tabor and Tabor 1984). The intracellular concentration of polyamines are generally highest in rapidly growing cells (Raina et al., 1980; Scalabrino and Ferioli 1982), and lowest in non-growing or quiescent cells (Heby and Andersson 1980). Spermidine may be released from cells when their growth is restricted by high density culture, or by inhibitors, or lack of growth factors, (Melvin and Kier 1980, Wallace and Keir 1981, Pegg and McCann 1982), and released from dead or dying cells, (Andersson and Heby 1980, Heby et al., 1980). Polyamine oxidase availability in mice sera, (Barford 1980) in horse and human sera (Barford 1980, Gaugas 1980, Allen et al., 1979) have been reported to be minimal and thus not eliciting the strong reaction produced by FCS on polyamines. However Higgins et al., (1969) have reported that horse serum contained more polyamine oxidase than FCS.

The interaction of polyamines (spermine and spermidine) with foetal calf serum in in vitro cultures and their effects on the inhibition of labelled precursor uptake in DNA synthesis have been shown by Byrd et al., (1977), Allen et al., (1997-1979), Gaugas (1980), and on the the inhibition of cellular growth (Higgins et al., 1969), (Katsuta et al., 1975) and (Maurer and Maschler 1979). Enzymic degredation of polyamines are required in order to elicit the suppression of normal and tumour cell proliferation, (Bachrach et al., 1967), (Higgins et al., 1969), and (Bachrach 1970). Polyamine oxidase (E.C.1.4.3.4.) oxidatively deaminates both ends of spermine but only the aminopropyl end of spermidine to produce the aminodialdehyde and aminomonoaldehyde respectively, (Tabor et al., 1964), (Bachrach and Reches 1969), (Morgan 1980), (Gaugas 1980), (Seiler et al., 1981), and

as shown by Allen et al., (1979) that the inhibitory effects of polyamines were dependent upon the presence of either polyamine oxidase or FCS. The results in Tables 3.1.1A to 3.1.4B, and Tables 3.2.1A to 3.2.3C confirm and extend the results shown by Byrd et al., (1977), Allen et al., (1977-1979), Maurer and Maschler (1979) and Smith et al., (1983) on the inhibitory effects of polyamines in in vitro cultures on a variety of cell types.

The interaction of spermine or spermidine with FCS did inhibit the uptake of 125IUDR by cell cultures of plasmacytoma, mammary adenocarcinoma and normal non lectin-stimulated thymocytes from mice and rats. The amount of isotope uptake inhibition by cell cultures, has markedly increased, as the pre-incubation period of polyamine-FCS has increased as described in Figure/Table 3.1.3A and 3B, which was a similar observation to that described by Bachrach and Reches (1966). The inhibition produced by the reaction of spermine with FCS was stronger than that produced by spermidine with FCS, as the dose of polyamine required to produce the ID50 (50% inhibition) of isotope uptake by the respective cell cultures was smaller for spermine than spermidine as described in Table 3. The ID50 for spermidine on plasmacytoma and adenocarcinome (0/4 incubation sequence, table 3 section 3.1) was similar at around 16uM, whereas for spermine, the adenocarcinoma was about 3 times more sensitive to its inhibitory effects, than plasmacytoma.

The thymocytes were more sensitive to the inhibitory effects of oxidised polyamine than the plasmacytoma or mammary adenocarcinoma described in Table 3, and this contrasts with the results of Allen et al., (1979) who have shown that the ID50, (spermine or spermidine) was higher for untreated normal rat thymocytes than for the leukemia and lymphoma cell lines. However, in only one experiment, described in

Figure/Table 3.3.1A and 1B, the thymocytes were more resistant than plasmacytoma to the inhibitory effects of oxidised polyamine as described in Table 3, section 3.3; thus the inhibition, depended on the culture incubation sequence. Rijke and Ballieux (1978) have shown that in invitro cultures of mouse thymocytes which were in spontaneous DNA synthesis were insensitive to the inhibitory effects of oxidised spermine or spermidine, with a similar finding by Allen et al., (1979) on rat thymocytes. The results described in Figures/Tables 3.1.1A to 3.3.1B show that mouse or rat thymocytes which were in spontaneous DNA synnthesis were sensitive to the inhibitory effects of oxidised spermine and spermidine.

However, Rijke and Ballieux (1978) and Allen et al., (1979) have shown that mitogen-stimulated lymphocytes and lymphoma cells in synchronous cultures (Allen et al., 1979), were inhibited by oxidised polyamines, thus implicating the ihibitory effects of oxidised polyamines on late G_1 phase or possibly at G_1/S interphase (Allen et al., 1979); which is closely related to polyamine biosynthesis, as the cellular levels of putrescine, spermidine and spermine progressively increase, as the cells traverse the cell cycle from G to mitosis, (Heby and Adersson 1980), (Boynton et al., 1980), (Heby 1981), (Pegg ad McCann 1982). Oxidised spermine has been shown by Bachrach et al., (1967) to inhibit protein and DNA synthesis, and to inhibit the growth of Ehrlich ascites after exposing the cells to oxidised spermine in invitro cultures. Oxidised polyamines (spermine and spermidine) can be converted into acrolein (Alarcon 1964-70), (Kimes and Morris 1971a), (Seiler et al., 1981). Kimes and Morris (1971b) have implicated oxidised polyamine and acrolein for the inhibition of nucleic acid and protein synthesis in E.coli.

However, the effects of the mono- and dialdelydes produced by the oxidation of spermidine and spermine were not cytotoxic as described in Table 3.5, and the inhibition of 125IUDR uptake was reversible, (Tables 3.4). Similarly, Byrd et al., (1977) and Allen et al., (1979) have shown that the effects of oxidised polyamines on their cell cultures were reversible and not cytotoxic.

Hussain et al., (1983) have shown that acrolein inhibited DNA synthesis and was cytotoxic for a 72-hour lectin stimulated rat thymocyte cultures, whilst oxidised spermine was not cytotoxic, yet inhibited 3H-Tdr incorporation at a much lower dose than acrolein. Preincubating the cultures with concentrations of acrolein in excess of 8uM for 3 hours prior to PHA stimulation, produced an irreversible inhibition of protein synthesis, due to its cytotoxicity, whilst a preincubation with FCS-spermine (50uM) for 24hours did not cause any inhibition of protein synthesis. Thus in a prolonged period of incubation, one would expect a sufficient amout of oxidised polyamine to be coverted to acrolein with ensuing cell death, yet as shown by Hussain et al., (1983) that during this prolonged incubation period in the presence of (50uM) spermine there was an insignificant change in cell viability.

If culture conditions and the incubation sequence were manipulated, the inhibitory effects of polyamines need not require FCS for its oxidation as described for plasmacytomas, (Figure/Table 3.2.1C) and mouse thymocytes, (Table 3.2.2C) which was a similar observation to that described by Smith et al., (1983) on the growth of lymphocyte and granulocyte colony cultures. Polyamine oxidase activity has been shown in cell homogenates by Quash et al., (1979) and Morgan (1980) and is produced by cells (Morgan et al., 1980), (Sunkara et al., 1981), and it is probable therefore that the source

of the enzyme is from the cells, which was oxidising the exogenous polyamines, as has been suggested by Smith et al., (1983).

Oxidised polyamines have been shown by Bachrach and Eilon (1967) and Eilon and Bachrach (1969) to cross link with DNA and form complexes with deoxynucleotides, deoxynucleosides, and as polyamines (spermine) has been shown to be required to stabilise DNA (McCormick 1977), (Gaugaus 1980), it may be that oxidised spermine may be competing or interfering with the function of native spermine (Allen et al., 1979), (Gaugas 1980). Polyamines in the presence of human serum were not inhibitory on isotope uptake by the respective cell cultures as described in Figures/Tables 3.1.2C, which was similar to the results of Allen et al., (1979) who showed that polyamines in the presence of either human or horse sera were not inhibitory as these two sera do not contain polyamine oxidase activity, (Allen 1979), (Smith et al., 1983).

All the sera which have been used for these experiments including bovine and human serum albumin were as effective as FCS on the inhibition of isotope uptake by cell cultures. There have been various reports in the literature on the effects of the above mentioned sera or their subfractions on their inhibitory effects on DNA synthesis and cell proliferation and growth. The effects of human serum and FCS were described by Curtiss and Edgington (1976); mice and foetal calf sera were described by Ablett et al., (1978); calf, FCS, horse and other sera by Harrington and Goodman (1980); mice sera by Chan (1971), Nelson (1972), Nelson and Shneider (1974), Beran (1974-1975), Metcalf and Russell (1976), Tomasi (1977) and Smith and Hammerström (1979); rat sera by Wilson (1973), Nadal and Bofa (1975), Nelson and Gatti (1976), Chan and Pollard (1978) and Zucker et al., (1981); human sera by Cooperband (1976), Chisari (1977), Curtiss and

Edgington (1977-1978), Green and Helson (1978), Chisari (1980), Douay et al., (1983), Szmitkowski (1983) and Cuthbert and Lipsky (1984). A variety of regulatory factors are present in serum and these have been reviewed by Cooperband et al., (1976), Nelson and Gatti (1976), Tomasi (1977), and Cathcart (1984) and some of which will be described as follows:

C reactive protein (CRP); a gamma migrating protein which attaches to cell membranes, inhibiting T cell rosette formation (Mortenson et al., 1975) and human mixed lymphoid reaction. The CRP is characterized by its ability to precipitate the C-polysaccharide of pneumococci in the presence of Ca^{2+} (Cathcart 1984).

Alpha fetoprotein; an embryonic glycoprotein which interacts with cellular membranes, inhibiting rosette formation of human lymphocytes with SRBC (Gupta and Good 1977). Alpha fetoprotein inhibits mitogen induced lymphocyte transformation (T & B cells) and mixed lymphoyte reaction cultures (Tomasi 1977).

Alpha-2-macroglobulin is a large molecular weight component of serum which is able to interact with proteases to suppress lymphocyte proliferation (Hubbard 1978). This macroglobulin has been demonstrated to be associated with lymphoid cells surfaces (McCormick et al., 1973) and probably may regulate immunological reactivity of these cells. High doses of alpha-2- macroglobulin have been shown by Ford et al., (1973) to suppress antigen induced lymphocyte proliferation, and by Streilein and Hart (1977) to suppress polycolonal B cell proliferation of a B cell line.

Human alphaglobulin described by Cooperband et al., (1976) was found to antagonize the blast transformation and its attendant increase in protein and DNA synthesis of human blood lymphocytes

induced by variety of antigens to which the donor was sensitive to, and by PHA stimulation or the mixed lymphocyte reaction, and suppressing the primary and secondary antibody response to sheep red blood cells in vivo. Cooperband et al., (1976) have termed their factor as Immunoregulatory alphaglobulin (IRA), with similar inhibitory effects on thymidine uptake by lectin stimulated lymphocytes or the mixed lymphocyte response and with immunosuppressive activity when assayed in vivo. The serum factor of Cooperband et al., (1976) was specific for lymphocytes, binds relatively weakly to a separate receptor and was not competing for lectin receptors and not cytotoxic. The globulin appeared to antagonize some early metabolic event in lymphocyte activation both in vitro and vivo. Harrington and Goodman (1980) have described a non-toxic factor present in normal sera of calf, foetal calf, human and rat which migrated with alpha-2-globulins, which selectively and reversibly inhibited the proliferation of cells in culture. DNA syntheses of normal rat hepatocytes, human lymphoblast lines and mitogen stimulated murine spleen cells were inhibited by (>90%), and to a lesser extent murine myeloma and human colon carcinoma. The active fraction which affected the transit of cells from G_1 to S was a protein that contained no lipoprotein. Harrington and Goodman (1980) concluded that their factor was not related to the lipoprotein, described by Leffert and Weinstein (1976), or the low density lipoprotein-inhibitor (LDL-In) that prevented mitogenesis of lymphocytes, which was described by Curtiss and Edgington (1976).

Several investigators have described the inhibitory effects of sera used in their experiments, on cellular proliferation and growth, and have described the effects of the lipoprotein fraction of serum as the factor responsible for this inhibition.

The lipoproteins are a hetrogeneous group of macromolecules which are classified according to their density, (VHDL, HDL, IDL, LDL and VLDL) (very heavy, heavy, intermediate, low and very low density lipoproteins) or according to their electrophoretic mobility as Pre-B, B and A. They are produced by the liver and the intestines, (LLoyd and Fostbrooke 1976),(Jackson et al., 1976).

The lipoprotein were shown to be involved in the inhibition of cell colony growth in in vitro cultures, as described by Chan (1971) and Beran (1975), both have used mice sera, which were shown to be inhibitory for mouse bone marrow cells colony formation, and the growth of granulocytic and macrophage colonies respectively. Chan (1971) and Beran (1975) have shown that the inhibitory activity was removed by ether extraction. Metcalf and Russell (1976) have also described the inhibitory effects of mouse serum on haemopoietic colony formation. Szmitkowski (1983) has described the inhibitory effects of human serum on granulocyte-macrophage colony formation, implicating the lipoproteins for this inhibition, and similarly Douay et al., (1983) have shown that human (LDL) inhibited the proliferation of normal human haempoietic progenitors in vitro.

Ablett (1978) have described the inhibitory effects of mice sera on the growth rate of sarcoma 180 and mastocytoma P-815 cell cultures, implicating the lipoproteins for this inhibition.

Ito et al., (1982) have shown that the growth rates of human FL, mouse FL, Swiss 3T3, and hamster embryo cells were inhibited by around 50% within 48 hours after introducing human (LDL) to these cell cultures which were preincubated for 24 hours before receiving the lipoprotein.

Nelson (1972) has shown that when mouse spleen cells were cultured in 5% mouse serum and stimulated by PHA, the uptake of tritiated thymidine by the spleen cells was depressed. Nelson (1972) has implicated the lipoprotein constituents of serum as the factors responsible for this inhibition.

The inhibitory effects on DNA synthesis by 5% mouse serum on mitogen stimulated lymphocytes and resting cells were also described by Nelson and Shneider (1974). These inhibitory effects were confirmed by Tomasi (1977) but with 10% mouse serum. Similar findings were reported by Smith and Hammerstrom (1979). DNA synthesis was depressed in mitogen stimulated rat lymphocytes when they were cultured with rat serum in concentrations above 5%, (Nelson and Gatti 1976).

The effect of rat serum lipoprotein on cell proliferation was described by Leffert and Weinstein (1976) who showed that very low density lipoproteins (VLDL) inhibited the pre-replicative protein synthesis required for the initiation of DNA synthesis in foetal rat hepatocytes cultured in lipoprotein deficient FCS, thus blocking the G_1 to S transit. Zucker et al., (1979) have shown that VLDL from normal rats inhibited DNA synthesis and protein synthesis in rat bone marrow cells, and similarly Zucker et al., (1981) have demonstrated that rat VLDL inhibited lymphocyte stimulation by mitogens. The effects of human lipoproteins were described by Curtiss et al. (1977) who showed that both human and murine low density lipoproteins (LDL) inhibited the two way mixed lymphocyte reaction using human and murine cells. The effects of human VLDL on the inhibition of the induction of DNA synthesis in human lymphocytes stimulated by mitogens and allogeneic cell stimuli were described by Chisari (1977) and Yi et al., (1981). Similarly Cuthbert and Lipsky (1984) have shown that

human LDL inhibited mitogen stimulated T-lymphcytes. The lipoproteins discussed above, have excluded the mention of FCS, and it may be reasonable to suggest that the lipoproteins in FCS may contribute in some comparable way to the inhibition of 125IUDR uptake by plasmacytoma, adenocarcinoma and murine thymocyte cultures, considering the following observations:

1. The lipoproteins have been shown to be species non specific in their action

a) Curtiss et al., (1977) have shown that human or murine LDL were effective in inhibiting the two way mixed lymphocyte reaction of human and murine cells.

b) VLDL from rats at an advanced stage of pregnancy have been shown by Chan and Pollard (1978) to be cytotoxic to rat prostate adenocarcinoma cells, hamster BHK21, human Hela cells but not foetal rat kidney cells

c) Ito et al., (1982) have shown that human (LDL) lipoprotein fraction was not specific in its action, as it inhibited the proliferation of human, mouse and hamster cells, but not the transformed cells lines of hamster.

2. Curtiss and Edgington (1977) and Curtiss et al., (1977) have shown

a) That human lymphocytes have receptors for (LDL-In) lipoproteins, and that both T and B lymphocytes were sensitive to LDL-In, (Curtiss and Edgington 1979). Similarly rat lymphocytes were shown by Zucker et al., (1981) to possess receptor for VLDL.

b) Curtiss and Edgington (1978) have shown that FCS at a concentration of 10% used in human lymphocytes cultures, did

compete with 125 Iodine-labelled human LDL-In for receptor sites on the lymphocyte surface; i.e.: there was a 64 % decrease in the number (125I-LDL-In) of labelled molecules per cell in the presence of FCS, as compared to cells cultured in lipoprotein-depleted FCS.

3. Vogt et al., (1969) have shown that transferrin isolated from FCS and cultured with mouse spleen cells, enhanced DNA synthesis, with a higher incorporation of tritiated thymidine, than when cultured with intact FCS. In contrast to whole FCS, there was less flattening of isotope uptake curve, with increasing amounts of foetal bovine transferrin within the range tested of 1 to 16%. At 16% FCS concentration, there was a depression of isotope uptake compared to 8% FCS concentration. Vogt et al., (1969) have suggested that FCS seemed to possess besides the stimulatory factor, another component which inhibited DNA synthesis. Vogt et al (1969) have tested mice sera, and were found to be inhibitory as well.

Cuthbert and Lipsky (1984) have shown that human LDL inhibited mitogen stimulated T lymphocyte DNA synthesis; and have also shown that the addition of transferrin reversed the inhibition of LDL containing cultures. Cuthbert and Lipsky (1984) have stated that both LDL and transferrin bind to their specific cell surface receptors, and enter the cell by similar means of receptor-mediated endocytosis, and concluded that LDL interfered with transferrin metabolism. Transferrin was shown by Rudland et al., (1977) to be essential for the cells to proceed to cell division, and was shown by Dillner-Centerlind et al., (1979) to be able to substitute serum as growth promoter for activation of T lymphocytes by mitogens.

4. Ito et al., (1982) have described the effects of human (LDL) lipoprotein fraction on the uptake of 3H-leucine, 3H-uridine and

3H-thymidine by human FL cells. The uptake of 3H-leucine was slightly suppressed during the first 9 hours, and the inhibition after 24 hours of incubation remained moderate. However, the inhibition of uptake of 3H-uridine and 3H-thymidine into the acid soluble and insoluble fractions was evident at 1 hour of incubation and progressively increased with time by 9 hours. After 24 hours of incubation the uptake of 3H-uridine recovered to moderate levels, whereas the uptake of 3H thymidine into acid insoluble fraction decreased drastically after 24 hours of incubation. Ito et al., (1982) have attributed this inhibition of incorporation of 3H-thymidine or uridine into acid insoluble fraction in the early period of treatment, mainly to the inhibition of uptake of the labelled compounds into the nucleotide pool, and in the case of 3H-thymidine there was a preferential inhibition of DNA synthesis at later periods of incubation. Ito et al., (1982) have also demonstrated that the rate of uptake of labelled thymidine or uridine within 1 hour of incubation was depressed as compared to control cultures without the lipoprotein fraction, and thus suggested, that the lipoprotein caused a decrease in the transport of these nucleosides across the cell membrane.

Ito et al., (1982) have also confirmed the early decrease of nucleosides transport across the plasma membrane, and showed that the intracellular metabolites derived from 3H-thymidine, in cells that were cultured with the lipoprotein fraction, were half less than that of cells cultured without lipoprotein. Thus as described in Table 1.5.1.1, the amount of the acid insoluble fraction decreased with increasing FCS concentration; with a similar decrease in the acid soluble fraction. The lipoprotein fraction may influence the cell membrane by raising the intracellular level of cAMP, and interfering with nucleoside transport across the membrane as Hauschka et al., (1972) have shown that the rate of uptake of 3H-Tdr into the acid

insoluble pool by Chinese hamster cells in the presence of Bu_2cAMP or Bt_2cAMP, [dibutyryl adensonsine 3':5'- cyclic monophosphaste] was inhibited by 50% (relative to controls) in the first 40 minutes of incubation, and with total inhibition at $\geqslant$10 hours of incubation. Hauschka et al., (1972) have also demonstrated in a different experiment to the one cited above, that the initial rate of uptake of 3H-Tdr into the acid soluble pool of hamster cells, cultured with Bu cAMP was inhibited by 84% (relative to controls) and concluded that the nucleoside transport was the primary process controlled by Bu cAMP, and that the labelling of acid insoluble material was a consequence of inhibited 3H-Tdr transport. Similarly, aminophylline (a drug which inhibits the action of phosphodiestrase, that degrades endogenous cyclic AMP) significantly decreased the pool size of acid soluble 3H-Tdr, and thus Hauschka et al., (1972) remarked that such a result should be expected if cyclic AMP were involved in the control of 3H-Tdr transport.

5. Ablett et al., (1978) have shown that the growth and multiplication of cell cultures of sarcoma 180 and mastocytoma P-815 was optimum when cultured with 10% FCS, however, when the concentration of FCS was increased to 15-20%, there was some inhibition in the growth rate. Ablett et al., (1978) have implicated the lipoproteins for the decreased growth rate.

6. Stimulated T lymphocytes secrete factors that contribute to the control of cell proliferation. Wolf and Merler (1979) have demonstrated the production by dividing human T cells, of an inhibitor of DNA synthesis and immunoglobulin synthesis. The inhibitor has been shown by Wolf and Merler (1979) to consist of two moieties, a non-dialyzable protein (which was inert with regard to inhibition of proliferation) and a polar lipid moiety, bound to the protein

fraction. The lipid protein, contained the inhibitory activity for inhibition of cell proliferation.

7. Schrier and Nordin (1977) have shown that the ability of different batches of FCS to support in vitro immunization of mouse spleen cells (PFC in vitro) was variable. Some batches were less supportive than others; even within the same (supportive quality) FCS batches, 10% FCS was more inhibitory for cell cultures relative to 5% concentration. Chisari (1980) has shown that human VLDL inhibited the primary haemagglutinin response of mice to SRBC (sheep red blood cells). Chisari (1980) has suggested that the immunoregulatory effect of human (VLDL) was caused by inhibition of early inductive events in the colonal expansion and entry of primed lymphocytes in the memory pool, and proposed a physiologic role for lipoproteins as growth regulators. The concentration required for complete suppression of lymphocyte proliferation was below or in the same range as their concentration in plasma. This suggested a normal suppressive role for lipoproteins in keeping the immune system in check (Cathcart 1984).

A regulatory role for lipoprotein in the sera of pregnant rats has been described by Chan and Pollard (1978), whose reconstitution studies with this type of serum has suggested that the cytotoxic activity associated with VLDL described earlier was usually masked by some inhibitors present in normal serum. However, when the rats were in an advanced stage of pregnancy the VLDL synthesis was enhanced and exceeded the masking effect of its inhibitor. The VLDL was heat resistant (56 ^{0}C) and to proteolytic enzymes.

Chan and Pollard (1978) have also suggested that the lipoproteins apart from being carriers for triglycerides, cholesterol and steriod hormones, may also contribute to surveillance against neoplasia like other non immunological host defences, which were reviewed by Apfel

(1976).

The lipoproteins may also be implicated in the interaction between macrophages, lymphocytes and tumour growth. Chapman and Hibbs (1977) have shown that the macrophage's cytotoxic effect could be inhibited by lipoproteins of the low density type, and have shown that the macrophage's cytotoxic ability can also be inhibited by enriching its membrane with cholesterol. Low density lipoproteins have a relatively high content of cholesterol and cholesterol esters, and have been shown by Bates and Rothblat (1974) to transfer free cholesterol to L cells. The rate of cholesterol synthesis in tumour cells is high (Howard and Kritchevsky 1969), (Chen and Heiniger 1974), and thus the lipoproteins can transport the extra cholesterol to the macrophage plasma membrane, and may modify the macrophage function.

Apart from the lipoproteins, sera contain a variety of factors which may influence cell proliferation. Honn et al., (1975) have shown that different batches of FCS were quite variable with regards to some of their constituents especially hormones. Hormones may exert their effects on target tissues through receptors on the target cell's membrane and cytoplasm (Malkinson 1978).

High concentration of estrogens were shown by Weichselbaum et al., (1978) to decrease the proportion of mammary carcinoma cells in S phase, either due to the relative prolongation of the other phases of the cell cycle, a decrease in the number of cycling cell or both. Similarly Lippman et al., (1976) and Riley et al., 1978) have shown that high concentration of estrogens, decreased the uptake by mammary carcinoma cells of 3H-Tdr into DNA, with cell death ensuing later. Clemens et al., (1979) have described the inhibitory effects of steriods on the uptake of 3H-Tdr by DNA synthesizing, mitogen

stimulated or MLC lymphocyte cultures. Progesterone (Clemens et al., 1979) reduced the incorporation of 3H-Tdr into the alcohol soluble pool which contains the phosphorylated nucleotides that may be incorporated into DNA, and the alcohol insoluble pool containing the 3H-Tdr that had already been incorporated into DNA. The inhibitory effects were reversible, and Clemens et al., (1979) have suggested that the reduction of 3H-Tdr incorporation into DNA might be secondary to a decrease in the amount of 3H-Tdr entering the cell.

Steroid hormones can influence several aspects of the function of the immune system. Some of these effects were reviewed by Stevenson and Fauci (1981) which included some selective effects on suppressor and helper T cells, the suppression of proliferation in the mixed leukocyte reaction, suppression of NK cell activity, and the stimulation of antibody production by B cells. The steroids in some systems, suppressed the production of lymphokines and suppressed its action on macrophages. The steroids also blocked the Fc receptor binding and function and depressed the bacteriocidal activity.

Prostaglandins are available in serum, and are secreted by stimulated macrophages (Bonney et al., 1978-1979). Upon stimulation the prostaglandins of the 'E' series are able to regulate immunological effector systems sych as lymphocyte mediated cytotoxicity and the inhibition of lysosomal enzymes release from polymorphonuclear leukocytes, possibly by increasing cAMP levels; (Metcalfe and Kaliner 1981), (Wolf et al., 1977)

Foetal sera contain an inhibitor of lymphocyte proliferation (Wolf et al., 1977), which is non-toxic, produced by unstimulated foetal monocytes, this factor was not produced by adult mononuclear cell cultures. Wolf et al., (1977) have postulated that this foetal factor contributes to the depressed maternal cell-mediated immune

response observed in pregnancy, besides the effects of thymus derived foetal suppressor cells which were described by Olding and Oldstone (1976). Nucleosides are present in sera and the uptake of labelled precursor by cell cultures for DNA synthesis can be influenced by serum, due to the presence of nucleosides in serum (Bernheim and Mendelsohn 1977). Carlton and Hamill (1977) have found that different batches of FCS at 10% concentration, contain between 0.6-8 uM of thymidine, and as described in Table 1.5.2, 10% FCS contains 3.4 uM of thymidine. The assessment of the amount of thymidine in serum was demonstrated in terms of a simple experiment, based on the dilution of label, which assumed that the inhibition of isotope uptake was mainly due to thymidine.

Unlabelled precursor, therefore, can compete with 3H-Tdr or 125IUDR to reduce the uptake of labelled precursors by DNA. Apart from dead cells in vivo which can contribute to the presence of thymidine in serum, macrophages are active secretors of thymidine because they lack thymidine kinase (Van der meer 1980). Macrophages and the lymphoid cells secrete a variety of substances which are present in serum, whose function is to contribute to the control of cell proliferation.

Thus nonspecific suppression of DNA synthesis assessed in terms of isotope uptake (125IUDR, 3H-Tdr) by humoral factors present in serum, should be viewed critically, and considered, when this in vitro system is used in assaying inhibitors of cellular proliferation.

A number of factors released by human (Green et al., 1970), (Hersh et al., 1974); by guinea pigs (Ernström and Nordlind 1977) rats and mice (Metcalf 1971), (Waldman and Gottlieb 1974), (Badger et al., 1974), (Bonmassar et al., 1978), (Jegasothy and Battles 1979), (Miyata and Kihara 1982) normal and stimulated lymphoid cells have

been reported in the literature, which were active in inhibiting the uptake of isotope by normal or tumour cells in vitro.

UIF (isotope uptake inhibitory factor), was produced by non stimulated spleen cells, thymocytes, lymph node and bone marrow cells. UIF is non species specific as it was produced by rat spleen cells and thymocytes, and mouse spleen, thymus, bone marrow and lymph node cells and was found to be active in inhibiting DNA synthesis of normal cell targets (mouse thymocytes) and tumour cells (murine plasmacytoma), in the presence or absence of FCS. The UIF described by Bonmassar (Bonmassar et al., 1978), was also produced by non stimulated cells and was not species or tissue specific. The production of inhibitory activity in UIF depended on the number of cells, cultured per petridish (i.e.: cell culture density), as UIF which was prepared from 25 x 10^6 cells per 5ml per petridish did not produce any significant inhibition even for a prolonged period of incubation as compared to UIF which was prepared from $5x10^7$ cells per 5 ml per petridish which produced a stronger inhibition of isotope uptake as described in Tables 2.2A1, A2 and A3. Similarly the strength of the inhibitory activity of UIF, described by Bonmassar (Bonmassar et al., 1978) depended on the culture density, with the inhibitory activity increasing as the culture density increased.

Hersh et al., (1974) have suggested that "cell overcrowding" in cultures i.e. cells cultures at high density in vitro, released substances which inhibited DNA synthesis. Green et al., (1974) have made a similar observation, to which Lopatin and Ranney (1977) have confirmed, adding that, with maximum cell to cell contact, their spleen cultures released a low molecular weight suppressor substance (LMWS) that inhibited DNA synthesis and decreased the mitotic index. Kasahara and Shiori-Nakano (1976) have also shown that peritonial

exudate cells cultured at high and low cell densities produced supernates with corresponding strength on the inhibition of DNA synthesis. Similarly, Lipkin et al., (1978), and Harel et al., (1984) have demonstrated that when cells were seeded at low density and allowed to proliferate until reaching stationary phase (contact inhibition), the cells released substances which inhibited cellular growth and DNA synthesis.

The UIFS which were produced by adherent and non-adherent cells were equally inhibitory. However when spleen cell cultures were depleted of macrophages by carbonyl iron treatment, no significant inhibition was produced by the macrophage depleted UIF preparation. The inhibitory activities of the adherent and non adherent spleen cells UIFS, described by Kasahara and Shiori-Nakano (1976) was stronger in the non adherent fraction than in the adherent fraction. The adherent cells are the macrophage enriched fraction, while the non-adherent cells are the lymphocyte rich fraction as has been described by Mosier (1967). The results described for the inhibitory effects of adherent and non adherent UIFS, and those reported by Kasahara and Shiori-Nakano (1976) are in contrast to the results described by Lopatin and Ranney (1977) who have shown that non adherent spleen cells produce relatively weaker UIF compared to adherent cell UIF which was more inhibitory, although the number of cells used for the preparation of adherent and non adherent UIFS by Lopatin and Ranney (1977) was equal, i.e. it was adjusted to 7, 5 or 3×10^6 cells per ml in each group, taking into consideration that adherent cells comprised about 30%, and non adherent cells about 60-70% of spleen cells, which were similar to the values described in this work.

In Kasahra's work (Kasahara and Shiori-Nakano 1976), the non adherent

fraction produced more inhibition of isotope uptake than the adherent cell fraction, as in those experiments the cultures were incubated according to their fractional content i.e. two thirds of the spleen white cell fraction were cultured as non-adherent and the remaining third as adherent, (Lopatin and Ranney 1977). Therefore the UIF of the non-adherent cell fraction of Kasahara and Shiori-Nakano (1976) was more inhibitory due to its higher cell culture density, but it should be noted that the inhbitory activity of the UIFS of the non-adherent spleen cell cultures reported in Tables 2.2A1, A2 and A3, and by Kasahara and Shiori-Nakano (1976) and by Lopatin and Ranney (1977) may have contained phagocytic cells which were moderately adherent, as it can be seen that the UIF produced by spleen cells depleted of macrophages by carbonyl iron and cultured at the same density of whole spleen STD UIF (standard UIF containing adherent and non-adherent cells) produced no significant inhibition of isotope uptake by plasmacytoma cultures even when assayed for an extended period (Table 2.2 A3).

Zipori (1980) has shown the mice spleen adherent cells retarded the growth of a Balb/c Abelson MuLv transformed cell line. Similarly, Zipori (1980) has also demonstrated that the growth of MPC-11 myeloma was inhibited when it was co-cultured with bone-marrow adherent cell fraction. UIF as described in Tables 2.2A1, A2 and A3 and 2.7 may contain several substances of high and low molecular weights such as the undialysable product $\geq 10^4$ daltons (Tables 2.2A1, A2, A3) and the amicon ultra filtrate which was less than 500 daltons (Table 2.7).

Similarly, the observation that cell culture supernates contained different substances was made by Fernbach et al., (1976) and Bonmassar et al., (1978). Bonmassar's UIF (Bonmassar et al., 1978) was partially sensitive to heat and proteolytic enzyme treatments as the

UIF inhibitory effects on 125IUDR uptake by leukemic cells was relatively reduced compared to the non-treated UIF. The STD.UIF, described in this work, was heat resistant and was relatively more inhibitory than its counterpart STD.UIF which was not heat treated, as described in Table 2.7. The difference between the respective inhibitory activities of the respective UIFS, may be examined further in terms of their effects on the amount of radioactivity available in the acid soluble and insoluble pools.

The UIFS produced by unstimulated spleen or thymus cells which were described by Miyata and Kihara (1979, 1982) were proteinaceous with a molecular weight of 11×10^3 to 14×10^3, that were released within the 3 hours of culturing, and which inhibited DNA synthesis of target cells by interacting with single-stranded DNA. The UIF (low molecular weight suppressor: LMWS) described by Lopatin and Ranney (1977), was released by unstimulated spleen cells after a 60-hour culture period, with a molecular weight between 500-800, which inhibited DNA synthesis and was not reversed by deoxycytidine. The UIF described by Kasahara and Shiori-Nakano (1976) contained low molecular weight substances some of which, identified as thymidine or thymidine monophosphate. Opitz et al., (1975a, 1975b), Evans and Booth (1976) and Fernbach et al., (1976) have shown that supernatants produced by cultures of spleen cells, thymus cells, peritoneal exudate cells or macrophages, contain low molecular weight substances that interfere with the uptake of isotope by cell cultures, but does not act as a true inhibitors of DNA synthesis with a consequence on cellular proliferation. Fernbach et al., (1976) have suggested that these supernatants may contain a true inhibitor of cellular proliferation, together with thymidine; a similar observation to that of Lenfant et al., (1973). Opitz et al., (1975b) have shown that macrophages can enzymatically degrade DNA of dead cells into

deoxynucleosides and deoxynucleotides.

Indeed, Staedecker et al., (1977) have confirmed and extended the observations made by Opitz et al., (1975b), and have shown that macrophages release substantial amounts of de novo synthesized thymidine, into their culture environment (0.4 - 1ug thymidine per 10^7 macrophages per 24 hours culture period), because macrophages lack the enzyme thymidine kinase (Stadecker et al., 1977), (Stadecker and Unanue 1979) and (Chan and Lakhchaura 1982). Apart from the secretion of thymidine, Chan and Lakhchaura (1982) have demonstrated that macrophage culture supernatants contain deoxycytidine, and thus the secreted deoxycytidine may counteract the growth inhibitory or cytotoxic effects of thymidine. Chan et al., (1974) have previously demonstrated that fibroblasts lacking thymidine salvage enzymes such as thymidine kinase, will release de novo synthesized thymidine. The UIF described by Bonmassar et al., (1978) had its activity reduced by 50% upon treating the spleen cell cultures with (Fudr) fluorodeoxyuridine. Fudr is taken up by the cell and converted into the monophosphate, which inhibits the endogenous production of thymidine by inhibiting thymidylate synthetase, which converts deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP), (Wheeler and Simpson-Herron 1973), (Adams et al., 1976). The inhibitory activity of spleen UIF, described by Bonmassar et al., (1978) was not impaired by immunosuppressive doses of x-rays, lethal for lymphoid cells in donor mice or in in vitro cultures. Neither was it impaired by an anti-macrophage agent such as silica particles, administered into donor mice prior to the culture of spleen cells in vitro. However, as described earlier in Tables 2.2A1, A2 and A3 that the UIF produced by spleen cell cultures depleted of macrophages had no significant inhibition compared to standard UIF, which was not depleted of macrophages, on the uptake of 125IUDR by target cells.

Stadecker et al., (1977) have shown that the inhibitory effects of their macrophage supernatant on the uptake of tritiated thymidine by EL-4 leukemic cells could be reversed by the addition of deoxycytidine. Stadecker et al., (1977) have demonstrated that the dose of exogenous thymidine added to the EL-4 leukemic cells to produce 50% inhibition of cellular growth, was 1×10^{-6} moles.

The 50% inhibition of isotope uptake by plasmacytoma cultures in the presence of thymidine for a 4 hour incubation period, described in (Figure/Table 1.14) was 6.6 x 10^{-7} moles of thymidine for cells cultured without FCS, and 3.2 x 10^{-7} moles of thymidine for cells cultured with 10% FCS. The total inhibition (≥90% of 125IUDR uptake was achieved at 1 x 10^{-4} moles of thymidine for both cultures with and without FCS. Reynolds et al., (1979) have shown that the growth rates of murine tumour cell lines (MPC11.45 and lymphoma EL.41) were inhibited by 50%, over a 3-5 day culture period with an exogenous dose of added thymidine of 1 x 10^{-3} and 1 x $10^{-4.9}$ moles respectively. Albert et al., (1982) have shown that the growth rate of a murine leukemic (L1210) cell line, was inhibited by 50% in a 48 hour culture period, with a concentration of 3 x 10^{-5} moles of thymidine.

Tsuboi and Kwong (1978) have shown that a dose of 1 x 10^{-2} moles of exogenous thymidine, has fully blocked the growth of normal (HS) colonic cells and its tumour variant (HT-29), without selective resistance to the inhibitory effects of thymidine. However, Lee et al., (1977) have demonstrated that there was selective resistance to the effects of thymidine in the murine (MEF) cell line, where 60% of the tumour variant of the (MEF) cell line died after an exposure period of 24 hours to 4.1 x 10^{-3} moles of thymidine, while 6.6% died in the normal (MEF) cell line.

A prerequisite for DNA synthesis, is the availability of all four deoxyribonucleosides triphosphates. De novo synthesis of deoxyribonucleotides occurs by the reduction of the corresponding ribonucleotides by ribonucleotide reductase. Alternatively, an exogenous supply of deoxynucleosides such as thymidine (1-B-D-2-deoxyribofuranosyl thymine, or deoxythymidine) can be used by the cell for DNA synthesis through the nucleoside kinases. Thus thymidine is incorporated into the cell by its thymidine kinase system to produce the respective Thymidine-mono, di, and triphosphates,

$$\text{Thymidine} \rightleftharpoons \text{TMP} \rightleftharpoons \text{TTP} \rightleftharpoons \text{DNA}$$

(Cleaver 1967, Reichard 1978)

The inhibitory effects of UIF on DNA synthesis, described in this work, may also be due to elevation of the adenyl cyclase levels in the plasma membrane, which produces relatively higher amounts of cAMP. As a consequence of higher levels of cAMP, the thymidine kinase activity may be reduced, as has been described by Hauschka et al., (1972). Reduced thymidine kinase activity will influence DNA synthesis, and hence cell proliferation will be affected.

Exegenous thymidine causes an increase in the intracellular concentration of TTP pools and to a lesser extent of TDP and TMP pools (Reichard et al., 1961, Gentry et al., 1965, Morse and Potter 1965, Reynolds et al., 1979 and Albert et al., 1982), while the concentration of deoxycytidine triphosphate drops (Reichard et al., 1961, Lee et al., 1977, Reynolds et al., 1979, de saint Vinc al. 1980, Howell et al., 1980, Eriksson et al., 1984). High levels of exogenous thymidine produce high levels of (dTTP) deoxythymidine tri-phosphate which have a feed-back inhibitory effect on the conversion of cytidine di-phosphate (CDP) to deoxycytidine di-phosphate (dCDP); the (dTTP) binds to the ribonucleotide reductase,

thereby decreasing the (dCTP) pool; (Bjursell and Reichard 1973, Reynolds et al., 1979, Howell et al., 1980, Albert et al., 1982, Eriksson et al., 1984) and thus affecting DNA synthesis. Addition of deoxycytidine prevents the thymidine induced lowering of the dCTP pools and the block in DNA synthesis, (Morris et al., 1963, Howell et al., 1980, Eriksson et al., 1984). Cells deficient in the enzyme thymidine kinase were not inhibited by excess thymidine, which indicated that a phosphorylated derivative of thymidine acted as an inhibitor, (Morris and Fischer 1963). Apart from using deoxycytidine, to verify the presence of thymidine, one can use an isotope of low specific activity (Lederman et al., 1981) which will be sensitive to exogenous thymidine; as exogenous thymidine will be competing with labelled isotope, apart from diluting the labelled precursor pool. Some inhibitors of cellular proliferation were found to reduce the grain count of cell cultures incubated with 3H-Tdr, without affecting the labelling index of the same cultures such as the bovine spleen extract described by Diatloff et al., (1978). Similarly the labelling index of plasmacytoma cultures, cultured with UIF were not significantly different from cells cultured without UIF, although the distribution in the number of cells containing the relevant grain count interval has changed, especially in the densely labelled group as described in Table 2.5. This may show that some substance was competing with isotope uptake, but if that were the case, then, one would expect that the frequencies in the light and medium labelled groups would have also changed relative to cultures without UIF, but that was not evident in the results. A further experiment to be envisaged is to pulse the cultures with isotope of a relatively low specific activity (in the presence of UIF) and process the slides autoradiographically in such a procedure which would allow a low, yet reasonable amount of grains to be present in the control and

experimental cultures. Having established this procedure, then the inhibitory effect of UIF may be noticed easily, in terms of isotope uptake and hence, the effects on grain count. But one point which was evident about UIF, was that it did not alter the labelling index of cell cultures as compared to control cultures, cultured in FCS without UIF; i.e. there was not any effect on S-phase cells that may have been effected by UIF.

Low molecular weight suppressors (i.e. LMWS= low molecular weight UIF) of DNA synthesis which were not cytotoxic, produced by macrophages have been described by Waldman and Gottlieb (1973), Calderon et al., (1974) and Ulrich (1974). Similarly, the inhibition of protein synthesis by LMWS, have been reported for spleen cells UIF (Kasahara and Shiori-Nakano 1976), (Lopatin and Ranney 1977), and thymocytes UIF (Ulrich 1974).

The UIF described in Table 2.2C was not cytotoxic as judged by trypan blue exclusion test, and its inhibitory effects on isotope uptake by cell cultures were reversible, (Table 2.3). The passage of cells through S phase, to mitosis was not blocked as the metaphase index was slightly reduced in the presence of UIF (Table 2.4.B), but the difference was not significant relative to the metaphase index of control cultures . However, when plasmacytoma cultures were incubated with UIF in vitro, and thereafter injected into mice to assess their growth in the recipient's spleen in terms of Tumour colony formation (Tumour CFU-spleen); UIF was inhibitory as described in Table 2.6B. The inhibition of UIF treated cells on colony formation was quite marked, and was more than that produced by Ara/c treated cell cultures. The inhibitory effects of UIF on colony formation may have changed the homing patterns of the plasmacytoma cells, or UIF may contain some inhibitory substances which have selectively killed some

tumour cells, or have blocked these cells from proliferation, while the remaining cells which proliferated to form tumour colonies in the spleen , were resistant to the effects of UIF. The inhibitory effect of UIF may probably be due to an inteference with cellular metabolism, which was not associated with any membrane damage, at least, as assessed in terms of trypan blue exclusion ability, in in vitro incubation periods of up to 12 hours with UIF, as described in Table 2.2C. The inhibitory effects of UIF may be analagous to lymphotoxin-induced cytolysis, which required extended periods, frequently greater than 12 hours to produce its effects, (Evans 1982). Similarly the inhibitory effects in UIF may be analagous to lymphotoxin in terms of colony growth, as lymphotoxin was shown by Evans (1982) to retard colony growth of aL929 cells. Another example which may be relevant here, is the supernatant which was described by Jeffes and Granger (1975). The supernatant which was produced by mitogen stimulated human lymphocytes, contained a variety of factors, such as proliferation inhibitory factor (PIF), cloning inhibitory factor (CIF) and lymphotoxin (LT). The activities of these factors were present in the same supernatant, and the particular effect was shown to depend on the concentration of the medium and the type of target cells employed. The medium effects were: (1) cytotoxic, at high concentrations of supernatant; (2) growth-inhibitory, at intermediate concentrations; and (3) transient reversible effects on cell division at low concentrations. The UIF described in this work was not produced by mitogen-stimulated cells, but the example of Jeffes and Granger (1975) was cited, as it may be that cell cultures produce some inhibitory substance without the need for cell proliferation or stimulation, as described by Greene et al., (1981), Fleisher et al., (1981) and Nair and Schwartz (1982).

The inhibitory effect of UIF may also be analogous to that of interferon or Bt_2cAMP, in which growth inhibition is mediated through the inducation of protein kinase (Greengard 1978). Moreover, if UIF was increasing the intracellular levels of cAMP, then it may influence cell proliferation. Coffino et al., (1981) and Insel and Fenno (1978) have shown that Bt_2 cAMP decreased the levels of ODC and S-adenosylmethionine decarboxylase (SAMD) activities. ODC and SAMD are required for the production of polyamines and hence for cell proliferation as has been described earlier. Apart from the need for the assessment of activities of the polyamine biosynthetic enzymes, in relation to the inhibitory effects of UIF; RNA and protein syntheses must also be assessed, as UIF may have several effects on intracellular processes. UIF sensitive cells may be affected by UIF at some stages in their cell cycle. The UIF described by Bonmassar et al., (1978) was cytotoxic or cytostatic, inhibiting the cellular proliferation of hamster sarcoma, Balb/c carcinoma and Hela cells in vitro. Similarly, the spleen UIF (Chalone) described by Attalah and Houck (1976) was cytotoxic for (L1210) murine leukemic cells but not for normal lymphocytes. It is apposite here to point out, that the previous molecular weight estimates of lymphocyte chalones were between 10^4 and 5×10^4 while the new estimates are of low molecular weights between 500 and 2×10^3 as shown by Patt and Houck (1980).

Low molecular weight suppressor of proliferation, may be associated with larger molecules, as Allen et al., (1977) have demonstrated that a lymphocyte "thymic chalone" was a complex of spermine and a high molecular weight carrier molecule. Attalah and Houck (1976) have suggested that their spleen chalone was cytotoxic for $G_1 - G_0$ resting cells, because no cytotoxic effects were observed in in vitro cultures incubated with the spleen extracts for around 6 hours, while it was evident at later periods of 8 to 10 hours. It may

probably be that some of the constituents of UIF described in Table 2.6B have affected some G_1 or G_0 cells from forming spleen colonies. It can be seen (Table 2.2A4) that the inhibitory effects on 125IUDR uptake by STD.UIF, dialysed STD.UIF and freeze- thaw (F.TH.UIF) have slightly increased when the incubation period was increased from 2 to 4 hours in the presence of FCS, which may be due to an interference with the flow of cells from G_1 to S, as Allen et al., (1979) have suggested that the effects of oxidised polyamine is between late G_1 and early S phase. It is also evident, (Table 2.2A4) that the same UIFS referred to above when assayed without FCS maintained a relatively constant effect on uptake without an increase in inhibition on uptake when the incubation period was increased from 2 to 4 hours. It may be due to the interaction of FCS with any of the STD.UIF substances which may contain polyamines, producing relatively more inhibition than their counterparts cultured without FCS, although the cultures that were cultured without FCS, may also contain polyamine oxidase. Patt amd Houck (1980) have pointed out, that one of the problems in the identification of endogenous inhibitors, was that most tissues contain polyamines. The presence of spermidine in the JB-1 ascites chalone preparation was demonstrated by Barford (1978) and Barford and Schjerbeck (1982).

The polyamines in the presence of FCS can produce the inhibitory mono and dialdehydes, or without the requirement of FCS if the polyamine oxidase enzyme is available in the culture supernatant as has been pointed out earlier. It can be seen, (Tables 3.1.3A and 3B) that when plasmacytoma cells were cultured with 0.8ug/culture with either spermine or spermidine in the presence of FCS, there was an increase in the inhibition of 125IUDR uptake by cell cultures by a factor of 6 for spermidine, and 3 for spermine when the incubation period was increased from 2 to 4 hours before pulsing with 125IUDR.

Similarly, the inhibitory effects of polyamine were dose dependent and more marked when incubated with FCS, but were dose independent and nearly constant, when incubated without FCS, as described in Table 3.2.1C.

The freeze thaw (F.TH. UIF) has produced a significant inhibition of isotope uptake in an extended incubation period (Tables 2.2A1, A2, A3) and the inhibitory substance which was released, did not depend on active cellular metabolism for its production. The action of F.TH.UIF was selective, while it inhibited the uptake of 125IUDR by plasmacytoma cultures, it stimulated the uptake of isotope by normal thymocytes (Tables 2.2B1, B2, B3) in the presence or absence of FCS, although being higher in cultures devoid of FCS. The stimulatory effects on DNA synthesis of murine thymocytes by F.TH.UIF may be analagous to the effects produced by lymphocyte activating factor (LAF), or interleukins 1, 2 and 3 as described by Maizel et al., (1981), Altman (1981), Larsson (1982), Oppenheim and Gery (1982), Garland (1982), Watson and Prestidge (1983) and Wood (1984).

The freeze thaw extract from macrophages (Waldman and Gottlieb 1973), and the spleen freeze thaw extract described by Lopatin and Ranney (1977) did not inhibit DNA synthesis, while the freeze-thaw spleen cell UIF described by Bonmassar et al., (1978) was of marginal inhibitory activity. Similarly, the freeze-thaw extract of Kasahara and Shiori-Nakano (1976) was of marginal inhibitory activity, when the cell concentration used to prepare the extract was 2.5×10^6 cells/ml. But when the cell concentration was raised to 5×10^6 cells/ml, the extract was inhibitory. The differential effects of F.TH.UIF, (Tables 2.2A1-A3 and 2.2B1-B3) needs to be investigated further. Cell culture supernatants of macrophages or of cell cultures that contain macrophages, contain a variety of substances that may be produced by

macrophages , some of which may be thymidine, hydrolytic enzymes, complement components, arginase, prostaglandins, polyamines and polyamine oxidases. Arginase has been shown to suppress the in vitro generation of cytotoxic response in the mixed leucocyte culture (Kung et al., 1977), and to suppress the growth of tumour cells in in vitro cultures (Currie 1978, 1980), (Van der Meer 1980) and (Schneider and Dy 1985). Arginase depletes arginine from the medium, as arginine is required by in vitro cultures for optimum growth (Eagle 1959). Arginase is also present in foetal calf sera and its concentration varies among different batches of FCS (De Laiter and Kihara 1968), and is relatively heat resistant up to 60 °C, for 1 hour. Bona and Chedid (1976) and Goodwin et al., (1978) have demonstrated that human blood mononuclear cells liberate prostaglandins 1 and 2 into the culture medium, and when the cell cultures were depleted of adherent cells the amount of released prostaglandin was reduced to an insignificant amount.

Prostaglandins (PGE2) have been demonstrated by Gery and Davies (1979) to act as an antiproliferative agent in lectin stimulated thymus or spleen cultures. The addition of indomethacin to macrophage-containing cultures, prevented the production of prostaglandins (Gerry and Davies 1979), thus indomethacin can be used to verify the source of inhibitory material in cell culture suprenatants (Lederman 1981). Caution should be used when interpreting data of inhibitory effects from culture systems in which macrophages, spleen cells or products thereof, contribute to the inhibition of DNA synthesis or cellular proliferation. This caution must also be viewed in terms of the effects which may be contributed by FCS, and the culture incubation sequence. The influence of FCS on the effects of UIF on the control of isotope uptake by cell cultures, together with the effects of culture incubation sequence, have been

described in the tables of section 2 of chapter 3. The effects of culture incubation sequence on isotope uptake were also evident in some of the results of the polyamine experiments described in section 3 of chapter 3.

If the properties of spleen UIF described in the tables of section 2 are taken into account, it may be difficult to assess the possible similarities or differences of UIF with any of the "isotope uptake inhibitory factors" described in the literature. The UIF described by Metcalf (1971), which inhibited bone marrow colony formation, was dialyzable, heat resistant and species non-specific. It was produced by cultures of normal bone marrow, spleen, lymph node and thymus cells, as well as by leukemic cells. The production of UIF by splenocytes in in vitro cultures, described by Bonmassar et al., (1978) was not impaired when donor mice were pre-treated with (IDF) immunodepressive factor that impairs T-dependent allograft reactions, and UIF was also produced by Balb/c nude splenocytes that lack T cells. All the UIFs which were described in this work (except the muscle cell line UIF), have been produced from cells of the central and peripheral lymphoid tissues. A variety of soluble mediators produced by mitogen stimulated and unstimulated human peripheral blood mononuclear cells have been reported by Green et al., (1981) and Fleisher et al., (1981) to inhibit mitogen-induced T cell proliferation and pokeweed mitogen stimulated B cells. The soluble suppressor factors described by Shou et al., (1980) and Nair and Schwartz (1981, 1982) were produced from unstimulated peripheral blood lymphocytes, and were shown to have suppressor activity towards lymphocyte responses to mitogens, and alloantigens in MLR and towards NK and ADCC functions. Nair et al., (1981) have also demonstrated that a certain fraction of mouse thymocytes was able to suppress the cytolytic activity of NK cells against their target cells in in vitro

cell cultures. These observations imply that lymphocytes or their products may contribute a role in normal immunologic homeostasis. It may be, therefore, that the inhibitory effects of UIF may contain a factor or factors which may contribute to the control of lymphocyte proliferation or effector functions. The inhibitory effects of UIF, therefore, must be examined further, in terms of their effects on lymphocyte proliferation and effector functions. The UIF was also produced by tumour cells in in vitro cultures, and was also found in the plasma of tumour bearing mice, (Bonmassar et al., 1978). UIF, therefore, may contribute at least in part, to the decline of tumour cell proliferation in lymphoma bearing mice close to leukemic death (Hofer and Hughes 1970).

As stated earlier UIF may contain a variety of substances which may contribute to the inhibition of isotope uptake, protein synthesis or cellular proliferation, in a specific or nonspecific way, as the UIF which was described by Miyamoto and Teryama (1971) produced by liver cells which inhibited the uptake of isotope by tumour cell cultures, contained thymidine hydrolase, an enzyme which can convert thymidine into thymine, and thus influencing the uptake of isotope by cell cultures. The BC3H-1-muscle cell line UIF inhibited the uptake of isotope by plasmacytoma cultures or thymocytes as described earlier, (Tables 2.2A and 2.2B), and therefore the BC3H-1.UIF may contain thymidine or some other substance which was contributing to this nonspecific inhibition, as the target cells and source of UIF were different types of tissues. That does not imply that the effects of spleen UIF, lymph node UIF, thymus or bone marrow UIF were specific, because each of the above UIFS was able to inhibit the isotope uptake of plasmacytoma cultures or thymocytes, as has been described earlier (Table groups: 2.1, 2.2, 2.3). Lopatin and Ranney (1977) have shown that their spleen macrophage rich fraction LMWS UIF

was species independent, effective against B and T precursor cells, whereas non lymphoid cells were unaffected by concentrations that produced a 50% inhibition of lymphoid targets.

A regulatory role has been suggested by Lopatin and Ranney (1977) for their LMWS, which was produced by unstimulated cells. When the cells were stimulated for example in an MLR culture, the production of LMWS decreased, but coinciding with peak proliferative activities, the levels of LMWS in stimulated cells began to reapproach levels of unstimulated cells and thus Lopatin and Ranney (1977) postulated a feedback regulatory control on lymphocyte division, which involved a sequential signal mechanism between activated lymphocytes and adherent cells or macrophages. Some of the contributions of macrophages to the regulation of cell proliferation were referred to earlier in the introductory chapter.

A regulatory role for adherent cells was suggested by Zipori (1980) for the inhibition of growth of plasma cell tumours by bone marrow adherent cells which were effective against the mature types rather than immature cells (in terms of their differentation pathway), that is, according to Zipori the bone marrow is a site for the proliferation of lymphocyte precursors rather than for accumulating of mature lymphoid cells. Zipori (1981) has shown that cell to cell interaction was required between adherent bone marrow cells and the MPC-11 myeloma, rather than a substance secreted by the adherent cells.

Bonmassar et al., (1978) have shown that when 2 types of lymphoma cell lines were co-cultured in vitro with spleen cells collected from lethally-irradiated donors compatible or not for the Hh-H2 complex, the uptake of 125IUDR by the tumour cells was inhibited, relative to control cultures incubated without spleen cells. The results

described by Bonmassar et al., (1978) using humoral factors from spleen cells or cell co-cultures to assess the inhibitory effects on tumour proliferation, were an in vitro correlate to results obtained earlier by Bonmassar and Cudkowicz (1976) and Campanille et al., (1977) which showed that normal haemopoietic or lymphomatous cells do not proliferate in the spleen of lethally irradiated recipient "nude" or normal mice incompatible for the Hh-locus. UIF produced by spleen cells may therefore contribute a role in the control of cell proliferation and inhibition. Apart from T and B cells, the spleen contains macrophages and natural killer cells (Stutman et al., 1980), (Herberman 1983), (Keller 1983), and these two cell types, contribute to the control of tumour cell proliferation in vivo host environments as shall be described in the section of tumour growth kinetics in normal and athymic "nude" mice.

The contributions of spleen cells, and cells of other lymphoid organs, or their products to the regulation or inhibition of plasmacytoma cell proliferation in invitro cultures, may have some in vivo correlates. One of these, is to assess the growth properties of plasmacytoma in two different immunological environments. But before discussing the growth properties of plasmacytoma in different in vivo environments, a short discussion on one of the techniques "the metaphase arrest" which is involved in kinetic studies, is appropriate, due to its usefulness in the study of tumour growth parameters.

The stathmokinetic or the metaphase arrest method has been a useful and a simple technique to determine the rate of entry of cells into mitosis or the cell birth rate, (Frei et al., 1964), (Tannock 1967), (Rolf Smith et al., 1974), (Riches et al., 1981) and (Jones

and Camplejohn 1983). Vincristine sulphate was used for the metaphase arrest experiment because it is a relatively better stathmokinetic agent than other agents such as colcemid, (Tannock 1967) and (Rolf Smith et al., 1974). The mice received an optimal dose of vincristine sulphate for a maximum period of 4 hours (Smith et al., 1974), and the number of metaphases was linear for this observation period, if a dose of a stathmokinetic agent were not optimal, and if the observation period were not optimal for the estimation of arrested metphases, metaphase loss will occur through incomplete arrest, or by degeneration (Tannock 1967), (Aherne and Camplejohn 1972), which will underestimate the mean birth rate (Wright and Appleton 1980) and therefore will overestimate the mean apparent cell cycle time (Tca).

In calculations of metaphase, or cell densities in tissue sections, corrections are required to allow for the overestimation of counts as fragments of these elements are included in the tissue section count.

Abercrombie, (1946) described a formula for correcting this overestimate, which included a relation between nuclear diameter, thickness of the tissue cross section, and the number of nuclei present in the section. The experimentally derived measurements of nuclear diameters in the tissue section are assumed to be equal to the mean random chord, which is equal to 0.79 of the value of the true diameter. However, true nuclear density estimates require a measurement of the true nuclear diameter. The value of the true diameters calculated assuming the experimentally measured diameters as equal to the mean random chord length, will in fact overestimate the true diameter. An over-estimate in nuclear diameter will result in an under-estimate in the nuclear density of the section, (Riches et al., 1981). Thus Riches (Riches et al., 1981) has introduced a correction,

to estimate true diameters from experimentally measured diameters, to derive correct nuclear density estimates. The values of the true average interphase and metaphase diameters which were determined with Riches's formula (Riches et al., 1981), were used in Abercrombie's correction (1946) to estimate the number of metaphases per unit volume and the number of cells per unit volume.

Moreover, by using the modifications of Riches 's group (Rolf Smith et al., 1974) in which the metaphase counts were related to the percentage of tumour cells available in each tumour zone (thus excluding the necrosis and space), the cell production rate per unit volume was calculated for tumour cells only. This is an accurate procedure, as the proportions of necrosis and space, may vary according to the zone, section, and tumour age, and consequently the non-necrotic or space areas will vary accordingly. The mean birth rate has slightly decreased as the tumour aged or increased in size from size 5 to size 8. The growth of the tumour in normal Balb/c mice, (Figure 4.2.1) was slightly convex upwards (towards the time axis) as the tumour volume increased to size 8, a sign of growth retardation. Tumour growth depends on tumour cell production (apart from cell input due to celular infiltrates) and cell loss. The tumour cell production rate is determined by the growth fraction (Ip) and median intermitotic cell cycle time (Tc) . As cell loss was almost equal at the three tumour volumes (sizes: 5, 6 and 8), and as the cell production rate was decreasing with increasing tumour volume, a reduced growth fraction or an increase in Tc , may be the contributors to the declining birth rate.

Wright (1975) has demonstrated that in the solid transplantable (Balb/c- mice) sarcoma , the birth rate decreased with increasing tumour age, with a relative decrease in the growth fraction; while Tc

remained nearly constant. Cell loss which increased with tumour age was a primary contributor to growth retardation. Similarly, Feaux De Lacroix and Lennartz (1981) have shown that in the solid transplantable (C3H-mice) mammary carcimoma, the Tca was increasing with tumour size or age, with a relative decrease in the growth fraction, an increase in cell loss, while the duration of Tc was not prolonged before the plateau phase of growth was reached. Frindel et al., (1967) and Lala (1977) reported a small decrease in the growth fraction, with a nearly constant Tc in a solid fibrosarcoma and an ascitic tumour growing in solid form, respectively. However, Simpson-Herren and Lloyd (1970, as described by Lala 1977) have shown that Tc has increased substantialy with tumour age, together with a reduced growth fraction in the transplantable sracoma 180, while in the transplantable plasacytoma, Tc was slightly increased together with a nearly constant growth fraction.

The cell production rates in the different zones (outer, middle and inner zones) described in this work were generally similar, apart from a small increase in the middle and inner zones relative to the outer zone. Tannock (1968) has demonstrated that the mitotic and labelling indices in a mammary tumour, were lower in tumour areas near necrotic regions and higher in regions near vascular supply, and accordingly the growth fraction was low in the former areas and high in the latter areas, while Tc was essentially the same for the two areas. Hirst and Denekamp (1979) have reported similar observation with their murine mammary carcinoma to that of Tannock (1968) with the new finding that Tc was higher in areas near necrotic regions. Wright (1975) has found evidence that the birth rate decreased gradually from the periphery to the centre of tumour, accompanied by a similar decrease in the growth fraction, although Tc remained constant in the three tumour zones. Similarly the cell birth rate and the growth

fraction, decreased progressively as the distance between the tumour cells and the blood vessel which supplied them, increased, (Jones and Camplejohn 1983). In the Balb/c tumours, reported in this study, the amount of necrosis was similar in all three zones of all respective tumours, apart from the "nude" mice tumours, which were relatively less necrotic.

The results described by Lauder et al., (1979) on their murine lymphoma were comparable to the results described in this thesis, in the sense, that the growth fraction in the inner and middle zones was slightly higher than the peripheral outer zone.

In general, as the tumour grows larger, restriction on the supply of nutrients and oxygen may become more limiting on cellular proliferation especially in the centre of the tumour, together with the accumulation of waste products, causes cells to cease proliferation and die. Apart from these factors, specific growth control mechanisms, may also be operating, such as chalones, which may contribute to the control of tumour growth, (Bullough 1977), (Rytomaa et al., 1977), (Laurence 1979) (Barford 1981) and (Barford and Schjerbeck 1982).

The differences between the weighted and unweighted regression analyses were small, in terms of the variability accounted for by the regression line. In the weighted model, there was a slight increase in the percentage variability accounted for by the regression line, (PVA), relative to the unweighted model tumours, except for the results of the "nude" mice, where the PVA has decreased relative to the unweighted regression. The variability in the metaphase collection function was due to two components: 1- Intertumour variability (as two tumours from two different animals were used for each metaphase determination in each time interval of observation).

2- The time variability, that is the number of metaphases increases with increasing time after vincristine injection.

As the variance of the metaphase count, is usually proportional to the mean metaphase count, it was considered to minimize this variation by weighting each mean metaphase count by the reciprocal of its variance. Wright and Appleton (1980) have suggested the weighted regression model was likely to reduce the variance of the turnover time obtained. The standard error associated with the respective Tca in tumour sizes 5, 6 and 8 in their respective zones in the weighted regression were slightly higher for tumour size 5 (and its respective zones), and slightly lower for tumours sizes 6 and 8 (and their respective zones), as compared to the unweighted regression. As for the tumours of the "nude" mice, the standard errors of the respective Tca were all higher in the weighted regression than their counterparts in the unweighted regression. Therefore the weighted regression was relatively beneficial for reducing the standard error in tumours sizes 6 and 8, but not for tumours size 5 and size 6 of the "nude" mice, together with the fact, that the cell production rates of all tumours in the weighted regression were less than that in the unweighted regression.

In the "nude" mice the birth rate in the unweighted and weighted regression analyses was higher than its counterpart (tumour size 6) in Balb/c mice together with a shorter Tca for the tumours of the "nude" as compared to normal Balb/c mice.

The cell loss factor was similar between the tumours of the "nude" and normal mice in the unweighted regression although in the weighted regression the cell loss in the tumours of the "nude" mice was slightly less. The higher cell production rate in the "nude" mice may be due to a higher growth fraction or a shortened Tc or a

contribution by both.

Rajewsky and Gruneisen (1972) have assessed cell proliferation of a transplantable rat mammary tumour in syngeneic rats in two immunological environments. One group of rats was treated with antithymocyte serum to make them deficient in T-lymphocytes, while the other group was untreated. Rajewsky and Gruneisen (1972) have found that the cell production rate of the tumour was similar in both environments, while the cell loss factor was higher in the normal rats than in the T-lymphocytes deficient group. The growth fraction and Tc were higher in T-cell deficient group, than in the normal group. The higher Tc in the T-cell deficient group was mainly due to a marked extention of the G_1 phase, relative to the normal mice.

The shortening of Tc in the untreated normal rats was interpreted as a selection against cells with long transit times through G_1. Further studies are required to assess the growth fraction and Tc with its phase duration and the immunological components that influence them, for the Balb/c plasmacytoma tumour growing in two different immunological environments, that of the athymic "nude" and normal mice.

The purpose of the study of tumour growth in "nude" mice and their normal counterparts Balb/c mice was to know whether differences existed in tumour growth parameters or not, as the transplanted plasmacytoma was growing into two different immunological environments. The thymus is absent in the "nude" mouse, with a consequent severe deficiency in T lymphocytes (Rygaard 1978) while their phenotypically normal littermates have a thymus together with the T lymphocytes.

One of the theories in tumour growth, is that transformed cells arise frequently during the life span of the organism, and that these cells are eliminated before clinical detection, by the defence mechanism of the host, known as immunological surveillance.

The concept of immunological surveillance against nascent tumour cells was formulated by Thomas (1959) and Burnet (1971) to account for an evolutionary mechanism that protected the host from aberrant cells, and as an explanation for the phenomenon in transplantation immunity, in which cellular immune responses were directed against foreign histocompatibility antigens. As T cell-mediated immune responses are involved in this process, immunosurveillance was viewed as antigen specific T cell-mediated process that led to the rejection of tumour cells, as these tumour cells expressed new antigens on their membranes, which are not present on their normal counterparts. Indeed T-cells contribute to the process of tumour rejection , and as will be discussed later, natural killer cells and macrophages contribute to the regulation of tumour cell proliferation.

This surveillance mechanism is asserted by the observations that : 1. Tumour cells have tumour associated or specific antigens which are distinct from those on the normal cells from which they arose. 2. The ability to induce an immune response against these antigens by thymus dependent lymphocytes, equivalent to allograft rejection. Thus the prediction of the surveillance mechanism is, 1: Tumours that grow despite immunosurveillance, are poorly immunogenic, and, 2: Any condition which is associated with a depressed T-cell mediated immunity, i.e. immunodeficiency disease, immunosuppression, immunologically priviliged sites, and age, should be associated with an increased tumour incidence.

Support for the immunosurveillance mechanism has come from evidence on allograft recipients, undergoing immunosuppressive drug therapy, who have been found to have a high incidence of tumours, the majority of which were of epithelial origin, although lymphomas were prominent also (Penn and Starzl 1972), (Green et al., 1981). In immunodeficiency states, there was an increased incidence of malignant disease mainly of the lymphoreticular system, such as Wiskott-Aldrich syndrome (Currie 1980), which is due to a loss of T-cells (Hood et al., 1978). Suppression of the immune system with antilymphocyte serum or neonatal thymectomy increased the incidence of tumours induced by RNA or DNA tumour viruses, (Law 1969).

The argument against immune surveillance was that there was no increased incidence of the majority of tumour types in immunosuppressed patients, (Moller and Moller 1978). Similarly mice made immunodeficient by thymectomy or irradiation, do not show an increased incidence of spontaneous or chemically induced tumours (Gillete and Fox 1975). Moreover, no spontaneous tumours appeared in "nude" mice, as described by Rygaard and Povlsen (1976) and Rygaard (1978), although Outzen et al., (1975) reported some increase in lymphoreticual tumour incidence in "nude" mice, but they have also shown that there was no increased incidence in "nude" mice compared to their heterozygate littermates, in sarcoma formation, induced by 3 methylcholanthrene. Also, there was no increased incidence of tumours in immunologically priviliged sites, (Moller and Moller 1978).

Prehn (1976) has shown that an extremely small inoculum of immunogenic tumour cells may grow when transplanted into syngeneic recipients, but a slightly larger dose may be inhibited. This "sneaking through" phenomenon has also been taken as an argument against T-cell mediated surveillance of small immunogenic tumour foci.

However, Gatenby et al., (1981) have suggested that "sneaking through" may result not from the failure to stimulate cytotoxic T-lymphocytes (TcL) but from the induction of suppressor (TsL) T-lymphocytes inhibiting the action of TcL lymphocytes. Also a very weak immune reaction (as the reaction must be, because it is directed against weakly immunogenic spontaneous tumour cells) may facilitate (immunostimulate) rather than inhibit tumour growth (Prehn 1976, Prehn and Outzen 1980). "Nude" mice which lack T-cells, have been shown by Kiessling et al (1975) to have high numbers of a cell type that showed cytotoxic activity against mouse leukemia cells, and was called "natural" killer (NK) cells. NK cells are present in mice apart from "nudes", humans and other animal species. These lymphoid cells, from normal mice which were not innoculated with tumour cells and other sources of antigen, had significant levels of cytotoxic reactivity against certain syngeneic or allogeneic tumour cells, (Herberman 1978). "Nude" mice were found to exhibit a high degree of resistance towards transplantation of a variety of syngeneic tumours, and therefore, alternative non T cell-mediated mechanisms have been suggested, to contribute a role in the protection against tumour growth, such as NK cells (Stutman and Lattime 1983) and macrophages (Keller 1983). The action of NK cells in vivo is direct and very rapid (within 4 hours) in the elimination of tumour cells labelled with 125IUDR. The elimination of isotopically labelled tumour cells was evident in normal mice (NK cells from high responder mice, more rapid than in low responder mice) and in "nude" mice, (Riccardi et al., 1980). The elimination of labelled tumour cells, correllated positively for in vitro and vivo assays, and for high responder mice the effectiveness for NK cells was more than that for macrophages. Similarly Riccarrdi et al., (1980) have shown that treating mice with pyran copolymer 2-3 days before the assays enhanced the NK cell

reactivities, but a marked decrease in the levels of NK reactivities have been noticed at seven days time when higly activated macrophages were detected. Riccardi et al., (1980) have argued that a possible role for TcL can be ruled out since in this system there was not sufficient time for sensitization, and since similar results were obtained in "nude" mice.

Alternative approaches to describe the effectiveness of NK cells against tumour growth in "nude" mice were reported by Habu et al., (1981) who have shown that upon depleting the NK cells in "nude" mice with selective antibody (anti-asialo GM1), the "nude" mice showed increased susceptibility to transplantation of syngeneic tumours. Adoptive tranfer of lymphocytes enriched for NK cells and depleted of T and B cells, into irradiated recipient mice has been shown to enhance the resistance of the recipient animals to growth of NK sensitive tumours (Herberman 1983). Karre et al., (1980) using beige mice (C57BL/6-bg/bg) which have a pronounced defect in NK cells, with no effect on T and B cells, have shown that the incidence of tumour formation by low dose inocula of chemically or virally induced leukemia was significantly increased, and the latent period decreased in bg/bg mice as compared to their normal (bg/+) normal heterozygote littermates.

Macrophages may also contribute to tumour surveillance, as Greenberg and Greene (1976) have reported a T-cell independent rejection of a low cell dosage of several tumour lines inoculated subcutaneously into syngeneic mice. The type of the cell involved in this T-cell independent rejection process was shown by Chow et al (1979) to be macrophage rather than NK cell dependent.

From the above discussion, the results that are described in Figure/Table 4.2.1 confirm the belief that resistance to tumour growth can be contributed to, by a non T-cell mediated response. The most obvious feature was the latency period of the "nude" mice tumours, which was twice as long as for normal Balb/c mice, and as the resistance weakened, the tumours eventually grew at a relatively faster rate in "nude" mice as compared to normal Balb/c.

The difference in the latency period may be accounted for, by the higher levels of NK activity in the "nude" mice than in normal Balb/c mice (Herberman and Holden 1978) and due to the antagonistic relationship between the presence of a thymus and the levels of NK cells in the animal, (Gidlund et al 1980). Gidlund et al, (1980) have also stated that the thymus was generating cells that suppressed the activity of NK cells.

Small and Trainin (1976), and Gabizon et al., (1976) have demonstrated that the anti-tumour effects of splenic T cells in tumour bearing mice were gradually dominated by a population of T cells which stimulated tumour growth, as the tumour progressed in growth. Umiel et al., (1978) have demonstrated the existence of two subpopulations of thymocytes in mice which retarded or enhanced tumour growth. Small et al., (1979) have postulated that thymocytes of tumour bearing animals, might be released prematurely into the spleen, where they may be involved in contributing to the inhibition of an anti-tumour response; thus enhancing tumour growth. A certain fraction of thymocytes which inhibited NK cell activity against its target cell, was described by Nair et al., (1981), and was shown to contain high amounts of (TdT) terminal deoxynucleotidyl transferase activity, a marker enzyme for precursor T cells or immature thymocytes. The TdT containing thymocytes may be similar to the thymocytes described by

Small et al., (1979).

The kinetics of target cell elimination are very rapid in the NK cell system, and with this virtue, it fits the description of a cell involved in surveillance, rather than the relatively slower T-cell response in target recognition and elimination. The latency period extention in the "nude" mice may also be related to the remarkably effective macrophage function against target cells. Zinkernagel and Blanden (1975) have demonstrated, the effective bacteriocidal activity of peritoneal macrophages against listeria in "nude" mice which was about twice as effective within the first 30 minutes after injection, as their normal littermates.

Meltzer (1976) has also demonstrated that macrophages from conventionally housed "nude" mice were highly cytotoxic against tumour monolayers, and were about twice as cytotoxic as macrophages from their normal littermates. However, Meltzer (1976) had also demonstrated that the cytotoxicity of macrophages from germ-free "nude" mice were similar to their normal littermates. Meltzer (1976) concluded, that macrophage activation in "nude" mice was dependent upon environmental stimuli, and added, that the presence of activated macrophages in untreated "nude" mice was consistent with the very low incidence of spontaneous tumours observed in these mice. The macrophage can achieve target cell destruction by surface to surface contact, and by the release of humoral mediators, such as UIF which has been described earlier.

Summary and Conclusion:

Cell proliferation kinetics of plasmacytoma has been assessed in vitro and in vivo.

The proliferation of plasmacytoma and other cell cultures in vitro has been assessed in terms of the culture environment, and humoral mediators (UIF) produced by cells of the central and peripheral lymphoid tissues.

Foetal calf serum (FCS), has been demonstrated to influence DNA synthesis as assessed in terms of isotope uptake by cell cultures.

The amount of radioactivity in the acid soluble and insoluble pools, was reduced as the concentration of FCS in the culture was increased. The relative change in isotope availability in both pools was approximately similar. The reduction of isotope activity in the acid soluble pool may suggest that FCS was interfering with the transport of isotope across the cell membrane; and the reduction of isotope activity in the acid insoluble pool may be a consequence to the effects observed in the acid soluble pool.

The inhibition of isotope uptake by FCS may depend on several factors, such as, the batch of FCS, the type of cell culture under investigation, its culture density, culture incubation time and culture incubation sequence.

FCS may interact with other substances available in the culture environment, either by increasing or decreasing the relative amount of isotope uptake by the respective cultures. The uptake of isotope by cell cultures that secrete polyamines into their culture environment which contains FCS, will consequently be reduced. The inhibitory effects of some UIF preparations on the uptake of isotope by cell

cultures were modulated by FCS, and by the culture incubation sequence. The inhibitory effects of some of the fractions of UIF at some incubation sequences (with FCS), may be regarded as either "false positives" or the inhibition is a transitional one in the sense that it depended on the incubation sequence. Thus the interpretation in the change of radioisotope uptake must be viewed critically. UIF may contain heat sensitive substances , as the inhibitory effect of UIF 56C remained stable in both FCS environments and this may suggest that some factors in UIF which were heat sensitive were reacting with FCS to influence the inhibitory pattern of radioisotope uptake. Similarly the inhibitory effects of polyamines without FCS on the uptake of isotope by cell cultures, were evident when the culture incubation sequence was changed.

Supernatants (UIF) produced by cells from the spleen, lymph nodes, bone marrow and thymus, were found to inhibit the uptake of isotope by cell cultures. The UIFS, which were obtained from the spleen, have demonstrated that the macrophage was the main source for the production of the inhibitory supernatants. The inhibitory material may contain a variety of substances, apart from thymidine, which may contribute to the regulation of cell proliferation.

The growth kinetics of plasmacytoma in vivo, in two different immunological environments have revealed that tumour growth may also be controlled in T-cell deficient environments, as the latency period of tumour growth, was longer in the "nude" mice than in the normal counterparts Balb/c mice. Natural killer cells and macrophages may be some of the contributors to the suppression of tumour cell proliferation in the earlier periods of tumour growth.

The growth kinetic studies of plasmacytoma in vivo, have demonstrated that the tumour birth rate was decreasing with increasing tumour age. Cell loss was approximately similar at the different stages of tumour growth, thus the growth fraction and the average intermitotic time may be influencing the cell production rate.

The birth rate was assessed in terms of a weighted and unweighted least square regressions, to compare the reductions in error variability associated with the metaphase "Collection Function" curves, and its consequent effects on the standard errors of the respective Tca associated with each birth rate curve. In some birth rate curves the variance was generally proportional to the metaphase index, but in others the variance was not proportional. To reduce the error variability associated with the birth rate curves, other types of transforms may be required apart from the variance weighted transform.

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